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Abstract

Title of Thesis: Regulation of Epidermal Growth Factor Receptor Signaling by cbl-b.

Name: Seth Ettenberg, Ph.D. 2001

Thesis directed by: Stan Lipkowitz, M.D., Ph.D. Investigator, National Cancer Institute, Associate Professor of Medicine and Molecular and Cell Biology Program, Uniformed University of the Health Sciences

Negative regulation of the Epidermal Growth Factor Receptor (EGFR) signaling is essential to proper regulation of cellular function. Genetic evidence from *C. elegans* and *Drosophila melanogaster* has demonstrated that cbl proteins are negative regulators of the EGFR in these organisms. To determine the role of mammalian cbl proteins in EGFR signaling, stable clones overexpressing cbl-b were created in two cell lines which have distinct biological responses to EGFR activation. In the 32D/EGFR murine hematopoetic cell line, overexpression of cbl-b inhibits Epidermal Growth Factor-induced (EGF) proliferation. In the MDA-MB-468 human breast cancer cell line, EGFR activation induces apoptosis. Overexpression of cbl-b in these cells inhibits EGF-induced apoptosis. These data demonstrate that the mammalian cbl-b protein, like the *C. elegans* and *Drosophila* homologs, inhibits EGFR function. The molecular basis of this inhibition was studied in these two model systems. cbl-b is phosphorylated and recruited to the EGFR upon activation. In both cell lines, activation of the EGFR and activation of multiple downstream pathways have a shortened duration of signaling when cbl-b is overexpressed. Further biochemical analysis demonstrated that cbl-b increased

activation-induced downregulation of the EGFR by enhancing EGFR ubiquitination and EGFR degradation. Specific inhibitors of either lysosomal or proteasomal proteases blocked cbl-b mediated EGFR degradation. Further analysis of cbl-b expression after cells are stimulated with EGF demonstrated that cbl-b is coordinately degraded along with the EGFR. Both EGFR and cbl-b downregulation requires an intact Tyrosine Kinase Binding and RING finger domain of the cbl-b protein. Additionally, binding of cbl-b to the EGFR is required for either protein to undergo EGF-induced degradation. Other proteins which are recruited to the activated EGFR complex are also coordinately degraded with the EGFR and cbl-b. These data allow us to construct a model of the regulation of the EGFR by cbl-b. Upon activation of the EGFR, cbl-b is phosphorylated and recruited to the EGFR. Next cbl-b enhances ubiquitination and subsequent degradation of the EGFR and other proteins bound to it. Finally, these data demonstrate that the mammalian protein cbl-b, like the *C. elegans* and *Drosophila* homologs, inhibits EGFR function.

Regulation of Epidermal Growth Factor Receptor Signaling by cbl-b.

by

Seth Ettenberg

Thesis submitted to the Faculty of the
Molecular and Cell Biology Graduate Program of the
Uniformed Services University of the Health
Sciences in partial fulfillment of the
Requirements for the degree of
Doctor of Philosophy 2001

Dedication

It is with great joy that I dedicate this thesis and its completion to my brother Andy. Your ever-present spirit has guided me not only in this endeavor, but also given me hope to make a difference in the outcome of the battle you so valiantly fought.

Acknowledgements

I am truly grateful for the environment in which I was educated over the past few years. I am most thankful to my colleagues in the laboratory for their patience, understanding and ability in teaching me everything which has enabled me to do this work. I would like to acknowledge my mentor Dr. Lipkowitz for his precise guidance and selfless sharing of knowledge during the course of my studies. Especially I thank Dr. Keane for his mentoring in all aspects of science, including those aspects not written in the literature. Additionally, a special thanks goes to Marion Nau for her patient reading and editing of this manuscript.

To my family from whom I find constant support, thank you. To my parents, for without their support, both financial and emotional, this would not have been possible. Of course to my wife, whose loving embrace and spirit has given me drive, resilience, and purpose, I cannot find the words for my appreciation.

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Introduction

For multi-cellular organisms the precise regulation of proliferation, differentiation, and apoptosis is essential for successful development and homeostasis. Growth factors and their receptors play a critical role in regulating these processes [1]. The crucial role of growth factor receptor pathways is highlighted by the frequent transforming mutations found in these pathways [2]. Inappropriate activation by mutation or amplification of any component in the signaling pathway (*e.g.* the growth factor, the growth factor receptor, or downstream components) can result in malignant transformation. For example, the v-*sis* oncogene is the transforming protein of the Simian Sarcoma Virus and is derived from the Platelet-Derived Growth Factor (PDGF)[1] (its cellular homolog) by a recombination event [3, 4]. v-*sis* transforms cells by autocrine stimulation of cell growth through the cell surface PDGF receptor. Constitutive activation of growth factor receptors can also result in malignant transformation [5]. Transfection of NIH 3T3 fibroblasts with constitutively active mutants of the Ret tyrosine kinase receptor causes oncogenic transformation [2, 6, 7]. Constitutive activation of many growth factor receptors has been found associated with human malignancies including macrophage colony-stimulating factor receptor, c-kit receptor, fibroblast growth factor receptor, and EGFR (See below.) [2]. Finally, activating mutations in the downstream molecules associated with the growth factor receptors can transform cells [2]. For example, activating mutations of the Ras proteins, a family of small GTPases which relay signals from the growth factor receptors to the nucleus [8], result in malignant transformation [9, 10].

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[1] See appendix I for abbreviations.

The EGFR is implicated in numerous human cancers [11]. Since the discovery that the oncogenic gene contained in the Avian Erythroblastosis Virus, v-ErbB, is a truncated EGFR, the EGFR has been found amplified, mutated, or rearranged in many human tumors [12, 13]. For example, overexpression caused by gene amplification of the EGFR is a common event found in glioblastoma (around 50%) and patients with such EGFR amplification have a very poor prognosis [14]. Overexpression of the EGFR has been demonstrated in many other tumor types including breast, bladder, and ovary [11, 15-17]. Furthermore, commonly found mutations of the EGFR cause truncations of the protein similar to v-ErbB. These truncated receptors are constitutively active. The most common example of this type of truncation, EGFRvIII, is caused by an inframe deletion of exons 2 through 7 containing the extra-cellular portion of the EGFR [18]. EGFRvIII is found in gliomas, breast cancer, ovarian cancer, lung cancer, and prostate cancer but not in normal cells [19-22].

The ErbB Family of tyrosine kinase receptors is made up of four family members: EGFR (ErbB-1)[23], ErbB-2 (Her-2/Neu) [24], ErbB-3 (Her-3) [25, 26], and ErbB-4 (Her-4) [27]. Other members of this family of receptors are also frequently found amplified in human tumors, especially ErbB-2 [24a]. These transmembrane receptors have the ability to signal diverse biological responses including cell growth, differentiation, and apoptosis [13]. This diversity is achieved by several layers of signaling complexity. First, each member of the family has the ability to respond to multiple ligands. For example, the EGFR receptor can be activated by EGF, TGF-α, Neuregulin, and β-cellulin with each ligand giving rise to a distinct biological outcome. Second, each ErbB receptor can homodimerize or heterodimerize with other members of

the family [28]. Homodimerization or heterodimerization is dictated by the ligand and the other ErbB family member present [29]. For example, neuregulin binds to ErbB-3 and induces preferential dimerization with ErbB-2 which results in a very potent mitogenic signal. Neuregulin can also induce ErbB-3-EGFR dimers, but this results in a less potent mitogenic signal [30]. EGF, on the other hand, preferentially induces dimers of the EGFR with ErbB-2. Further diversity may be achieved by altering the complex of downstream molecules which bind to the activated receptors [13]. The duration that a particular downstream pathway stays active also may specify the desired outcome. For example, in PC12 cells a long active MAPK signal can cause neuronal differentiation, while a shorter active MAPK signal can cause proliferation [31]. Finally, the cellular response to a given ligand can change in response to the concentration of ligand present or to the total level of expression of the receptor at the cell surface. In epithelial cells and many tumors, EGF generally causes proliferation. However, in some epithelial tumor cell lines, which express very high levels of the EGFR (e.g. MDA-MB-468 and MDA-MB-431), EGF induces apoptosis [32, 33].

The EGFR is the prototypic member of the ErbB family (Figure 1). It is a type I transmembrane glycoprotein with a predicted molecular weight of approximately 140 kDa. However, the mature protein migrates on a SDS-PAGE gel at about 170 kDa due to glycosylation [34]. The N-terminal 622 amino acids make up the extracellular portion of the receptor and contain the site of ligand binding. This domain contains 12 sites where N-linked glycosylation can occur and two cystine rich regions between which ligands have been demonstrated to bind. The extracellular domain is followed by a short α-helix (residues 623-644), which spans the plasma membrane once. The remaining 542 amino

acids of the protein are internal to the cell and consist of the domains responsible for initiating the cascade of signals and trafficking of the receptor. The tyrosine kinase domain, which spans residues 690-954, is highly conserved across all tyrosine kinase receptors and is essential for a signal to be transmitted by the receptor [35]. The C-terminal tail of the receptor has several sites for tyrosine phosphorylation, which play crucial roles in signaling and regulation of the receptor [36]. Several signal transduction proteins, containing the SH2 and the PhosphoTyrosine Binding domains utilize these phosphorylation sites as docking sites [36]. Additionally, the area between amino acids 984-996 interacts with actin filaments, structural components of the cytoskeleton [37]. Lastly, there is an internalization sequence from amino acids 954 to 991. This sequence enhances receptor endocytosis [38].

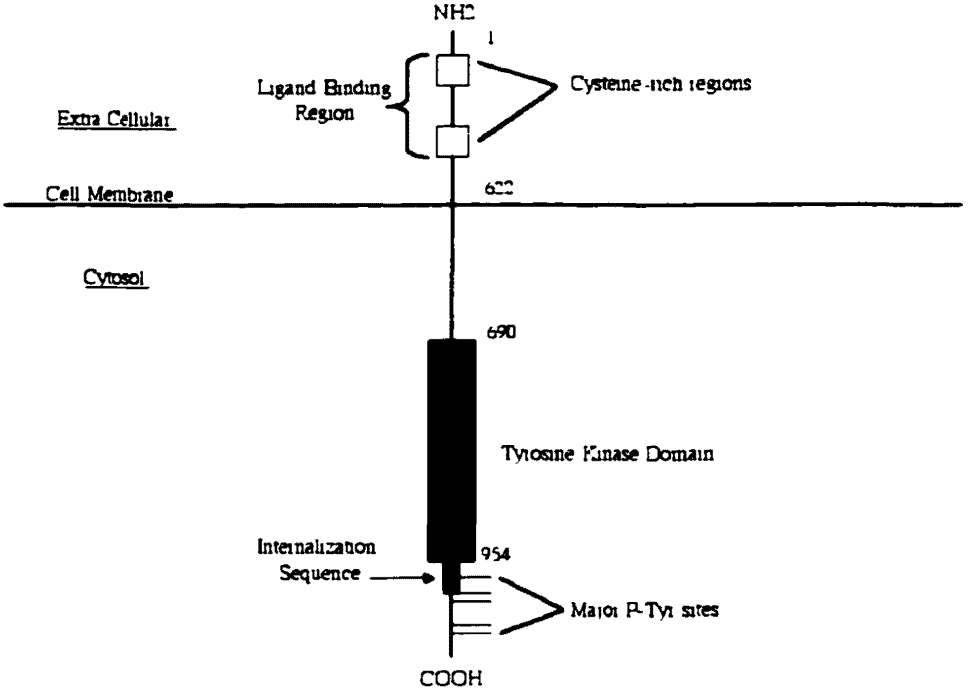

Figure 1: Schematic of the Epidermal Growth Factor Receptor. Cysteine rich regions are shown as open boxes, the tyrosine kinase domain is shown as a black box, the internalization sequence is shown as a blue box. The sites of tyrosine phosphorylation (P-Tyr sites) are indicated in the C-terminus of the receptor by lines. Numbers indicate amino acid residues.

Growth factor activation of the EGFR progresses through several distinct steps including: ligand binding, receptor dimerization, receptor activation, signal transduction to second messengers, and finally receptor signal attenuation. Ligand binding is the initial step in activation of the EGFR. Activation of the EGFR requires dimerization, as is the case for all known tyrosine kinase receptors. Upon ligand binding the EGFR receptor rapidly dimerizes with another EGFR (or another ErbB receptor) and these two receptor chains undergo transphosphorlyation [36]. Most growth factor ligands of the tyrosine kinase receptors are themselves bivalent and can therefore bind two receptor chains and dimerize them. However, EGF molecules exist in a monomeric state and are thought to bind to one receptor chain. The exact mechanism of EGFR dimerization is still not completely understood. It has been suggested that ligand binding may cause a conformational shift in the receptor that changes the affinity of the receptor for neighboring ligand bound receptors [39]. However, recent data suggest that EGF molecules may be bivalent, with both a high and low affinity binding site. Thus, as for other growth factors, one EGF molecule may bind to two ErbB receptors and cause them to dimerize [40]. This dimerization is responsible for and essential to the tyrosine kinase activity of the receptor. Dimerization leads to activation of the receptor and phosphorylation of tyrosine residues [41-44]. The kinase activity is absolutely required to initiate the biological effects associated with the receptor (*e.g.* mitogensis, differentiation, survival, cytoskeletal changes, and apoptosis [45]. Experiments using receptors with mutations in the kinase domain have demonstrated a complete lack of phosphorylation and of subsequent downstream signaling [36, 46].

The next step is signal transduction and the recruitment of kinase substrates and signaling proteins to the activated receptor [36]. Multiple signaling pathways are affected by EGFR activation. One major pathway activated by the EGFR is the MAPK pathway [34, 47]. In this pathway, activation of the EGFR results in recruitment of GRB2, which binds the EGFR via its SH2 domain at phosphotyrosine residue 1068. GRB2 has been shown to bring SOS and GAP, (the GTP-GDP exchange protein for Ras), to the EGFR and this in turn results in Ras activation [48]. This is followed by MAPK activation and activation of transcription factors which leads to cell growth [49]. In addition to affecting cell growth, EGFR activation of other pathways such as PLCγ, AKT, and STAT can lead to morphologic changes, changes in cell motility, and differentiation in cells stimulated by EGF [50-55].

The final step in EGFR signaling is termination of the signaling pathway. In general, there are two established mechanisms to attenuate GFR signaling. Receptors can directly activate phosphotyrosine phosphatases. Phosphatases reverse signaling through the receptor by removing the phosphate from the specific activation related tyrosines on the receptors (or on the downstream molecules) [56]. For example, the phosphatase SHP-1 dephosphorylates the EGFR at tyrosine residue 1173 and may negatively regulate signaling through the receptor [57]. Alternatively, receptors can be removed from the cell membrane via endocytosis. During endocytosis receptors disassociate from their bound ligand and are either recycled to the cell membrane or degraded in late endosomal compartments by lysosomal or proteasomal proteases [58]. This second process of receptor signal attenuation is termed downregulation and has been observed for many different growth factor receptors [58]. Endocytosis and degradation of the ligand bound

EGFRs has long been documented by many groups [59, 60]. Upon ligand binding, EGFRs cluster in clathrin-coated pits and are internalized. Internalization can cause as much as 80% of the original surface ligand binding sites to be lost after a short period of EGF stimulation [61]. Both proper EGFR conformation and kinase activity are required for this process since truncated receptors and receptors with inactive kinases are not downregulated [58, 62, 63]. Malignant transformation of cells by mutant EGFRs which do not undergo downregulation highlights the importance of downregulation as an attenuation mechanism. For example, truncation of the EGFR at amino acid 973 (which removes the internalization sequence) results in a receptor which does not undergo downregulation and transforms NIH 3T3 cells [64].

cbl proteins are negative regulators of the EGFR [65]. v-cbl was first identified as the transforming gene of the Cas NS-1 murine retrovirus. This retrovirus induces pro-B-cell lymphomas and myeloid leukaemias in mice and also transforms fibroblast cell lines [66]. v-cbl (Casitas B-lineage Lymphoma) had no homology to other known oncogenes. v-cbl is a fusion between the viral gag protein of the retrovirus and the 355 N-terminal amino acid residues of the cellular protooncogene c-cbl [67, 68]. The N-terminal region of c-cbl is capable of transforming cells while full length c-cbl can not [69]. Since the discovery and cloning of c-cbl, other cbl family members have been found. These include two additional mammalian cbl genes: cbl-b, and cbl-3; one *Drosophila* gene with two alternatively spliced isoforms: d-cbl$_L$ and d-cbl$_S$; and one *C. elegans* gene: sli-1 (Figure 2). This unique family of proteins is highly conserved from nematodes to mammals [70]. All cbl proteins have a highly conserved N-terminal region. This region includes a novel N-terminal TKB domain, which recognizes a D(N/D)xpY motif [71].

This domain is made up of a four helix bundle, an EF-hand Ca^{2+} binding domain, and an SH2 domain [72]. Although the amino acid sequence is divergent from other classic SH2 domains, crystallographic studies reveal that the cbl TKB domain contains all the necessary structural requirements of an SH2 domain [73]. Also the N-terminal region of all cbl proteins contains a highly conserved C_3HC_4 RING finger. RING finger domains have been identified in many E3 ubiquitin ligase proteins [74, 75]. Structural studies have revealed that the conserved cystine and histidine residues present in the RING domain form a cross-braced structure which holds two zinc molecules in a coordination complex [75]. The C-terminal domains of the cbl proteins are more variable. All cbl proteins, except d-cbl_S, have a proline rich domain which follows the RING finger domain. In addition, the C-terminal portion of c-cbl and cbl-b contains the major tyrosine phosphorylation sites. Many SH2 and SH3 containing signal transduction proteins have been demonstrated to bind within the C-terminal domain. The Ubiquitin Associated domain is the most C-terminal domain and is present only in the long forms of the cbl family (c-cbl, cbl-b, and d-cbl_L). This domain occurs in a number of ubiquitin pathway proteins and has been suggested to help confer substrate specificity within the ubiquitin pathway [76]. Furthermore, this domain has been shown to mediate c-cbl homodimerization [77]. The RING finger domain, the proline rich domain, and the ubiquitin associated domain are deleted in the transforming v-cbl protein.

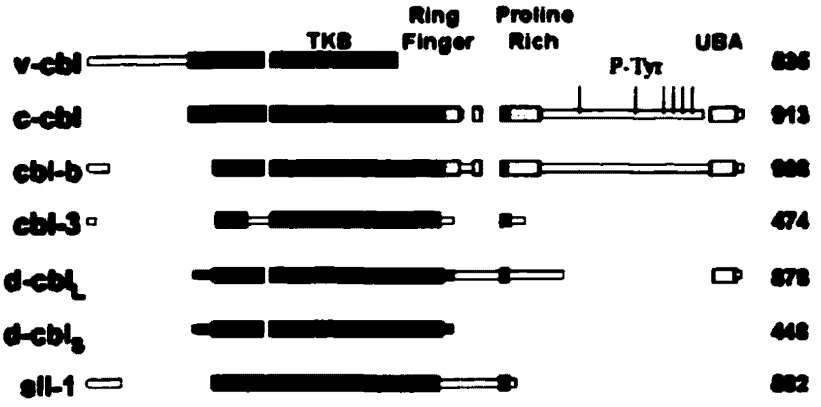

Figure 2: The cbl Family of Proteins. v-cbl: the GAG-cbl fusion protein of the Cas NS-1 murine retrovirus; c-cbl: the human protooncogene of v-cbl; cbl-b: the second human cbl protein; cbl-3: the third human cbl protein; d-cbl$_L$: the long form of the *Drosophila melanogastor* cbl protein; d-cbl$_S$: the short form of the *Drosophila melanogastor* cbl protein; sli-1: the *C. elegans* cbl protein. The red bars indicate the areas of homology conserved in all cbl proteins. The blue bars indicate the area conserved between v-cbl and c-cbl. The green bars indicate the areas conserved between d-cbl$_L$ and d-cbl$_S$. The gray bars indicate the areas conserved between c-cbl, cbl-b and d-cbl$_L$. The white bars indicate unique areas among the family. The tyrosine kinase binding domain (TKB), RING finger, the proline rich region, and the ubiquitin associated domain (UBA) are indicated above the diagram. Major Tyrosine phosphorylation (p-Tyr) sites are indicated with vertical lines. The total number of amino acid residues within each molecule are indicated to the right.

Initial biochemical evidence of the role of cbl proteins in tyrosine kinase signaling came from that c-cbl is rapidly tyrosine phosphorylated after T-cell receptor activation [78]. At the same time c-cbl was identified by a cDNA expression library screen using a GST fusion protein containing the SH3 domains of NCK [79]. NCK is an adaptor molecule involved in signaling by tyrosine kinase receptors [80]. Since the initial studies in T-cells, cbl proteins have been shown to become phosphorylated and, in some cases, to bind to numerous receptors upon ligand activation. These include the B-Cell, GM-CSF, IL-3, PRL, Fcγ, c-Kit, PDGF, and EGF receptors [80]. Additionally, cbl proteins have been shown to bind to numerous proteins involved in signaling by these

receptors including: Grb-2, Shc, PI-3K, PLCγ, Crk, Src, ZAP-70, Lck, Lyn, Fyn, Talin, and VAV [80]. The immense diversity of proteins and receptor pathways associated with cbl proteins suggest that cbl proteins play an important role in many signaling pathways.

Functional studies of the cbl proteins have demonstrated both positive and negative roles in signaling pathways. For example, Vitamin D treatment of osteoclasts induces bone resorption by activating the Src kinase, which in turn phosphorylates c-cbl. Downregulation of either the Src kinase or c-cbl function (by anti-sense oligonucleotides) inhibits vitamin D stimulated bone resorption [81]. This suggests that c-cbl plays a positive signaling role in this system. In contrast, a study from the laboratory of Lawrence Samelson demonstrated that overexpression of c-cbl in mast cells inhibits the activity of Syk kinase stimulated by the FcεRI receptor and prevents serotonin release [82]. These results show an inhibitory role for c-cbl in this pathway. The same functionally diverse outcomes seem to be true for cbl-b as well. Overexpression of cbl-b in T-cells causes the constitutive activation of cytosolic kinase ZAP 70 and NFAT, indicating a positive role in signaling [83]. However, a study by Barbacid and colleagues demonstrated that overexpression of cbl-b inhibits the EGF- or PDGF-induced activation of JNK [84]. Thus, in several distinct signaling pathways cbl proteins have been shown to have both positive and negative effects.

Developmental studies in *C. elegans* and *Drosophila melanogaster* have demonstrated that cbl proteins can act as inhibitors of EGFR mediated development [80]. Work from the laboratory of Paul Sternberg identified the gene suppressor of lineage defect -1 (sli-1 the *C. elegans* cbl homolog) in a screen for mutants which could restore normal development to worms with mutations in the let-23 protein (the *C. elegans* EGFR

homolog) [85]. Mutations in let-23 result in worms with a hypo-vulva phenotype. Null mutations in sli-1 restore normal vulval development in these worms. These genetic data demonstrated that sli-1 acts as a negative regulator of let-23. Further genetic experiments indicated that sli-1 functions at or near the receptor [86]. Additional evidence of inhibition of the EGFR function by cbl proteins came from two studies in *Drosophila* [87, 88]. In this system signaling through the *Drosophila* EGFR is critical for development of the R7 photoreceptor cells [89]. The first study demonstrated that overexpression of d-cbl (the *Drosophila* cbl homolog) can inhibit EGFR dependent photoreceptor cell development in the eye. The second demonstrated that d-cbl could bind to the *Drosophila* EGFR homolog. This further suggested a direct regulatory effect of cbl proteins on the EGFR.

Due to the genetic and biochemical evidence described above in *C. elegans* and *Drosophila* we hypothesized that mammalian cbl proteins regulate EGFR function. Our laboratory had recently cloned the cbl-b gene and began to address the question of whether cbl-b and its mammalian homolog c-cbl, might regulate EGFR signaling.

Hypothesis: cbl-b regulates EGFR signaling.

- Specific Aim #1: To develop model systems to study the role of cbl-b function in EGFR signaling.
- Specific Aim #2: To define the mechanism of cbl-b function on EGFR signaling.

Paper 1

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cbl-b inhibits epidermal growth factor receptor signaling

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The role of *cbl*-b in signaling by the epidermal growth factor receptor (EGFR) was studied and compared with c-*cbl*. We demonstrate *in vivo*, that *cbl*-b, like c-*cbl*, is phosphorylated and recruited to the EGFR upon EGF stimulation and both cbl proteins can bind to the Grb2 adaptor protein. To investigate the functional role of cbl proteins in EGFR signaling, we transfected *cbl*-b or c-*cbl* into 32D cells overexpressing the EGFR (32D/EGFR). This cell line is absolutely dependent on exogenous IL-3 or EGF for sustained growth. 32D/EGFR cells overexpressing *cbl*-b showed markedly inhibited growth in EGF compared to c-*cbl* transfectants and vector controls. This growth inhibition by *cbl*-b was the result of a dramatic increase in the number of cells undergoing apoptosis. Consistent with this finding, *cbl*-b overexpression markedly decreased the amplitude and duration of AKT activation upon EGF stimulation compared to either vector controls or c-*cbl* overexpressing cells. In addition, the duration of EGF mediated MAP kinase and Jun kinase activation in cells overexpressing *cbl*-b is shortened. These data demonstrate that *cbl*-b inhibits EGF-induced cell growth and that *cbl*-b and c-*cbl* have distinct roles in EGF mediated signaling.

Keywords: cbl proteins; EGF receptor; signal transduction

Introduction

The c-*cbl* proto-oncogene is the cellular homolog of the v-*cbl* oncogene, the transforming gene of the Cas NS-1 murine retrovirus, which causes pre B cell lymphomas and myelogenous leukemia in mice and transforms NIH3T3 cells (Blake *et al.*, 1993; Langdon *et al.*, 1989). The transforming v-*cbl* protein is a gag-v-*cbl* fusion protein containing only the N-terminal 40% of c-*cbl* (Blake *et al.*, 1993). It is believed that the transforming *cbl* protein acts as a dominant inhibitor of the normal *cbl* protein function (Miyake *et al.*, 1997). The c-*cbl* protein is phosphorylated upon activation of a variety of receptors which signal via protein tyrosine kinases (PTK) including the EGF, B-Cell, CSF-1, Fcγ, c-Kit, PDGF and T-Cell receptors (reviewed in Miyake *et al.*, 1997; Smit and Borst, 1997). c-*cbl* also interacts with the activated receptors and this interaction is mediated both by association with the adaptor protein Grb2 and by direct binding of a unique phosphotyrosine binding (PTB) domain in the N-terminus of c-*cbl* (reviewed in Miyake *et al.*, 1997; Smit and Borst, 1997). The c-*cbl* protein has also been shown to associate with a variety of SH2 and SH3 proteins involved in signal transduction including CRK, Fyn, Lck, NCK, PI-3-Kinase and Shc (Miyake *et al.*, 1997; Smit and Borst, 1997). Thus, c-*cbl* appears to be an important molecule involved in many signal transduction pathways.

Developmental studies in *C. Elegans* and *Drosophila melanogaster* have demonstrated that the *cbl* family proteins are able to act as inhibitors of epidermal growth factor receptor (EGFR) mediated development (Hime *et al.*, 1997; Meisner *et al.*, 1997; Yoon *et al.*, 1995). Genetic experiments in *C. elegans* have indicated that the function of the *cbl* protein is at the level of the receptor and the Sem5 protein, placing the *cbl* proteins at an early point in the signal transduction cascade (Jongeward *et al.*, 1995). In addition, one oncogenic form of c-*cbl*, 70Z-c-*cbl*, has been shown to stimulate the kinase activity of both resting and stimulated EGFR (Thien and Langdon, 1997). There are conflicting data in the literature that mammalian c-*cbl* protein may directly inhibit EGFR autophosphorylation (Thien and Langdon, 1997; Ueno *et al.*, 1997). The biological role of the normal c-*cbl* protein in EGF signaling remains to be elucidated.

We recently cloned human *cbl*-b, a homolog of the c-*cbl* proto-oncogene (Keane *et al.*, 1995). *cbl*-b shares several structural similarities with c-*cbl*, most notably in the N-terminal PTB domain, the C3HC4 zinc finger and the proline rich domain (Keane *et al.*, 1995). We have previously shown that *cbl*-b, like c-*cbl*, binds to a variety of signaling proteins *in vitro* via SH3 interactions (Keane *et al.*, 1995). Here we compare the role of these two mammalian *cbl* family members in signaling by the EGFR.

Results

Interaction of cbl-*b and* c-cbl *with the EGFR*

Other investigators have previously shown that c-*cbl* is phosphorylated and recruited to the EGFR upon EGF stimulation (Bowtell and Langdon, 1995; Fukazawa *et al.*, 1996; Galisteo *et al.*, 1995; Khwaja *et al.*, 1996; Levkowitz *et al.*, 1996; Meisner and Czech, 1995; Odai *et al.*, 1995a; Soltoff and Cantley, 1996; Tanaka *et al.*, 1995; Ueno *et al.*, 1997). To compare the interaction of *cbl*-b and c-*cbl* with the EGFR, HA-epitope tagged *cbl*-b or HA-epitope tagged c-*cbl* was transfected along with the EGFR into human embryonic kidney cells

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expressing the SV40 large T-antigen (293T). The EGFR was also transfected alone for comparison. The cells were then starved and stimulated with EGF and the resultant lysates used to compare the interactions between the EGFR and each *cbl* protein. These lysates were immunoblotted and probed with anti-phosphotyrosine (anti-pty), anti-*cbl*-b, anti-c-*cbl*, anti-EGFR and anti-HA (Figure 1a). Lysates from cells transfected with either *cbl*-b and the EGFR or c-*cbl* and the EGFR each showed prominent EGF-induced tyrosine phosphoproteins at ~180 kDa and ~120 kDa corresponding to the positions of the EGFR and the cbl proteins, respectively (Figure 1a, top panel). The unique phosphoproteins seen in the c-*cbl* and *cbl*-b transfected cells represent degradation products of the respective *cbl* proteins. This was determined by sequentially reprobing the blot with antibodies for c-*cbl* or *cbl*-b which recognize epitopes at the N and C termini of the respective proteins (data not shown). In contrast, the cells expressing the EGFR alone showed prominent phosphorylation of the EGFR but did not have the prominent 120 kDa corresponding to the *cbl* proteins (longer exposure did show a phosphorylated 120 kDa band corresponding to the endogenous *cbl* proteins). Cells overexpressing either *cbl*-b or c-*cbl* did not have consistent significant differences in the phosphorylation levels of the stimulated EGFR compared to each other or compared to cells overexpressing the EGFR alone (Figure 1a). Interestingly, 293T cells transfected with the EGFR alone consistently had significantly higher levels of phosphorylation of the unstimulated receptor compared to cells expressing the EGFR and either c-*cbl* or *cbl*-b (Figure 1a).

Several other observations may be made from the data in this figure. First, the anti-HA antibody shows that the transfected proteins are expressed at easily detectable levels. Second, *cbl*-b migrates at a slightly higher position than c-*cbl*. Finally, the lower band seen with the anti-*cbl*-b antibody is likely to be degraded protein since it is not seen with the anti-HA antibody (the HA epitope is on the C-terminus of the protein) nor when the protein is immunoprecipitated (see Figure 1b).

The lower panels in Figure 1a also suggest that the anti-*cbl*-b and anti-c-*cbl* antibodies specifically identify their respective proteins on immunoblots. However, longer exposure of the blots probed for c-*cbl* or *cbl*-b revealed expression of the endogenous c-*cbl* and *cbl*-b respectively in 293 cells at levels ~20 - 30-fold less than

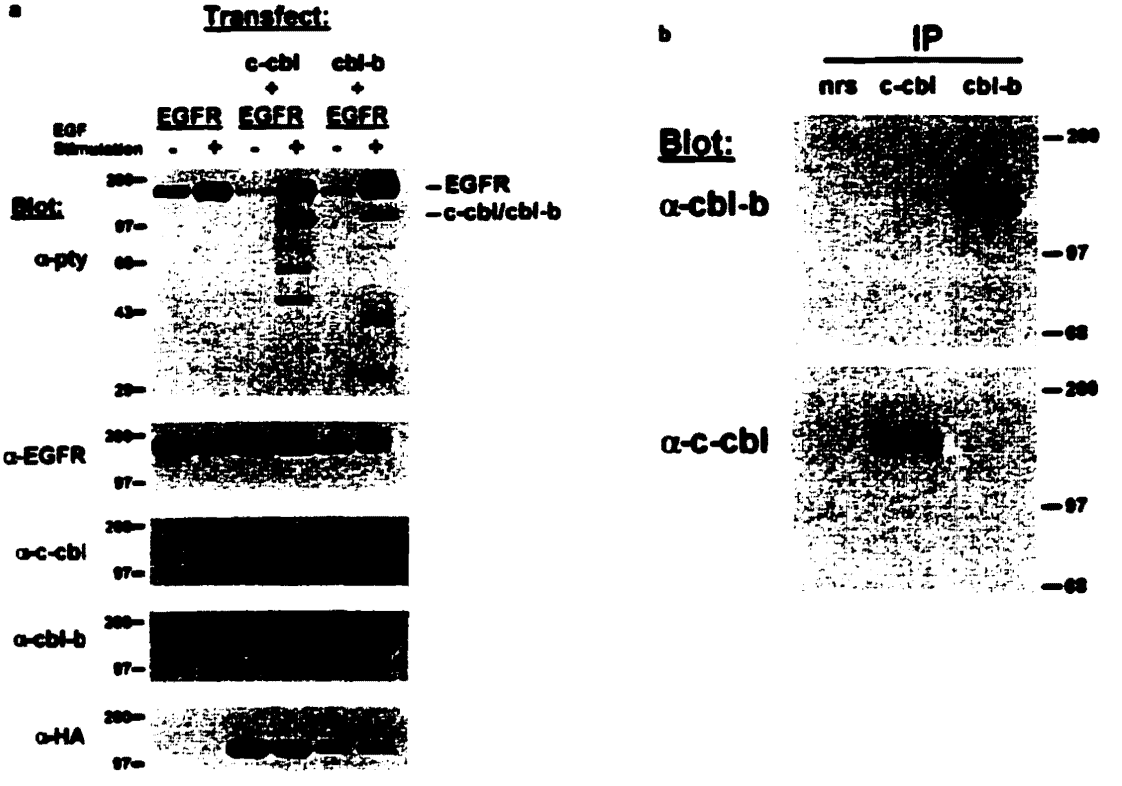


Figure 1 (a) 293T cells were transiently transfected with either the EGFR alone, HA tagged *cbl*-b and the EGFR, or with HA tagged c-*cbl* and the EGFR. The cells were starved for 4 h, incubated with or without EGF (100 ng/ml) for 10 min, and cell lysates were prepared. The proteins were separated by SDS PAGE on parallel gels, transferred and immunoblotted with either the anti-phosphotyrosine antibody (α-pty), the anti-EGFR antibody (α-EGFR), the anti-c-*cbl* antibody (α-c-*cbl*), the anti-*cbl*-b antibody (α-*cbl*-b), or the anti-HA antibody (α-HA) as shown to the left of the blots. The positions of the EGFR, *cbl*-b and c-*cbl* are shown to the right of the α-pty blot. Molecular weight standards (in kDa) are shown to the left of the α-pty blot. The middle three panels show expression of the transfected EGFR, c-*cbl* and *cbl*-b genes (α-EGFR, α-c-*cbl* and α-*cbl*-b respectively). The bottom panel shows the expression of c-*cbl* and *cbl*-b using the α-HA antibody. (b) Specificity of *cbl*-b (H121) and c-*cbl* (c-15) antisera. 293T cells were cotransfected with *cbl*-b and c-*cbl*. Lysates from these cells were immunoprecipitated with non-immune rabbit serum (nrs), anti-c-*cbl* antibody, or anti-*cbl*-b (antibody shown along the top of the figure). The immunoprecipitates were divided and run on two gels. One gel was immunoblotted with α-*cbl*-b and the other was blotted with α-c-*cbl*. Molecular weight standards (in kDa) are shown to the right of the blots

the transfected proteins (data not shown). This raised the possibility that the antibodies did have some cross reactivity. To confirm the specificity of the two antibodies for both immunoprecipitation and immunoblotting, *cbl*-b and c-*cbl* were cotransfected into 293T cells and lysates were immunoprecipitated with each antibody and with non-immune rabbit serum (nrs) (Figure 1b). Equal amounts of the precipitates were run on two parallel gels and then immunoblotted with anti-*cbl*-b and anti-c-*cbl* antibodies. The data shown indicate that both antisera are specific for both immunoprecipitation and immunoblotting. These data also indicate that there is no significant interaction between *cbl*-b and c-*cbl* since there is no evidence of co-immunoprecipitation of one with the other.

To further compare the interactions of *cbl*-b and c-*cbl* with the EGFR, we immunoprecipitated the EGFR from EGF stimulated 293T lysates containing either *cbl*-b or c-*cbl* (Figure 2, top panels). Both *cbl*-b and c-*cbl* (heavy arrows) were co-precipitated with the EGFR (light arrows) in the stimulated cells but not in the unstimulated cells. There was no difference in the kinetics of the recruitment to the EGFR at the time points analysed. When these immunoprecipitates were probed with an anti-phosphotyrosine (anti-pty) antibody, phosphorylated proteins were seen that migrated

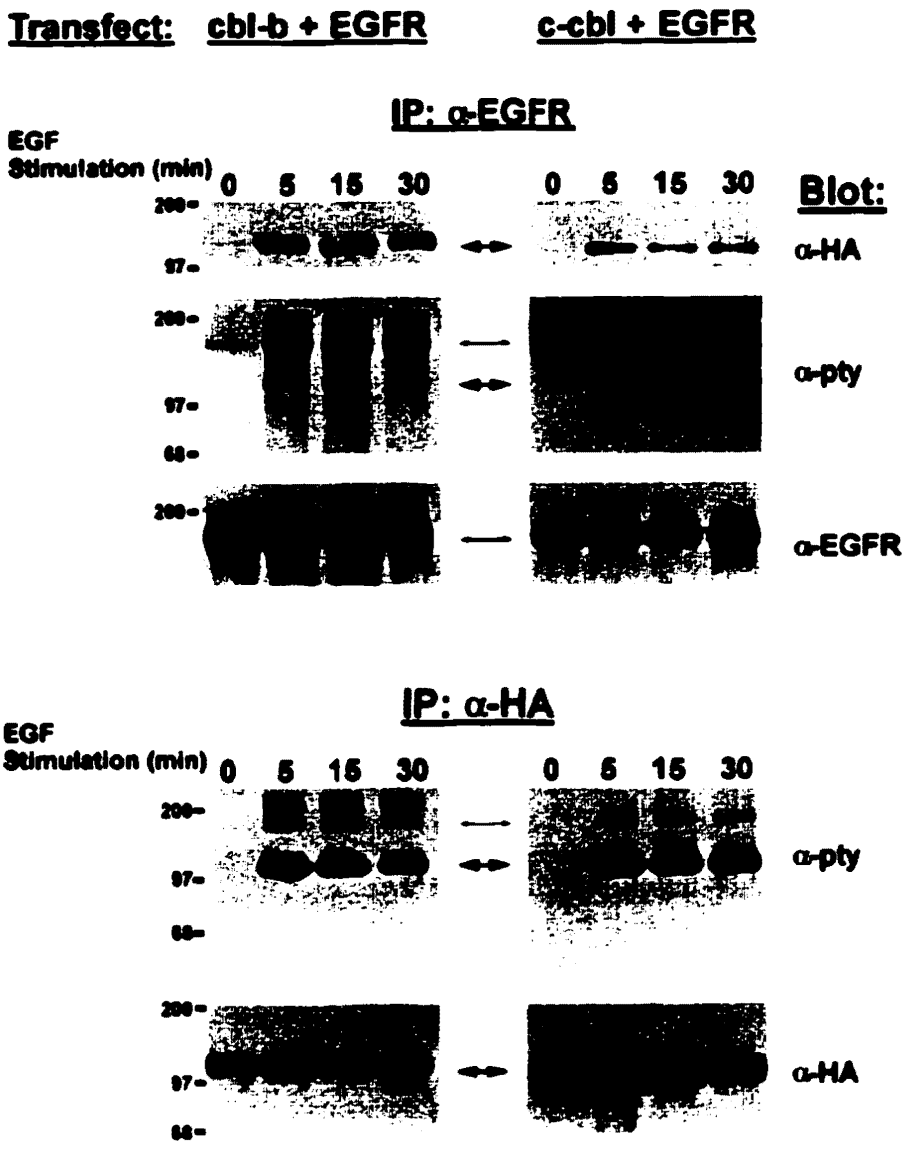


Figure 2 *cbl*-b and c-*cbl* are recruited to the EGFR and phosphorylated upon EGF simulation. (a) 293T cells were transiently transfected with either HA tagged *cbl*-b and the EGFR or with HA tagged c-*cbl* and the EGFR. The cells were starved for 4 h, incubated with or without EGF (100 ng/ml) for the times indicated along the top of the figure and then the cells were lysed. The EGFR (top panels) or *cbl*-b and c-*cbl* (bottom panels) were immunoprecipitated using the α-EGFR antibody or the α-HA antibody respectively. The precipitated proteins were run on parallel gels, transferred and immunoblotted with the antibodies shown to the right of the blots. The positions of the *cbl* proteins are indicated by the heavy arrows and the positions of the EGFR are indicated by the light arrows. Each protein (EGFR, *cbl*-b or c-*cbl*) was equally expressed in lysates from each time point (data not shown). Molecular weight standards (in kDa) are shown to the left of the blots

at the sizes of EGFR and *cbl* proteins. To confirm that *cbl*-b and c-*cbl* were phosphorylated upon EGF stimulation, the transfected *cbl* proteins were immunoprecipitated with the anti-HA antibody and probed with the anti-pty antibody (Figure 2, bottom panels). Both *cbl* proteins are phosphorylated upon EGF stimulation. A phosphoprotein that migrates at the size of the EGFR is also seen when the *cbl* proteins are immunoprecipitated. Together these data demonstrate that both *cbl*-b and c-*cbl* are similarly phosphorylated and recruited to the EGFR upon EGF stimulation.

c-*cbl* is recruited to the activated EGFR predominantly through its interaction with the adaptor protein Grb2 (reviewed in Miyake *et al*., 1997; Smit and Borst, 1997). To test whether *cbl*-b also interacts with Grb2, 293T cells were transfected with the EGFR, *cbl*-b and the EGFR, or c-*cbl* and the EGFR and then the cells were starved or stimulated with EGF. The respective *cbl* proteins were immunoprecipitated with the anti-HA antibody and the precipitates were probed for Grb2. Grb2 co-precipitated with *cbl*-b and c-*cbl* from both starved and EGF stimulated lysates (Figure 3, top panel). Only slight enhanced binding of Grb2 was seen in the *cbl*-b precipitate from EGF stimulated cells and no EGF enhanced binding was seen between Grb2 and c-*cbl*. The anti-pty blot demonstrated that the precipitated *cbl* proteins became heavily phosphorylated upon EGF stimulation and that they associated with the EGFR as demonstrated below (Figure 3, middle panel). The *cbl* proteins were equally precipitated by the anti-HA antibody (Figure 3, bottom panel) and the lack of Grb2 in the precipitates from the cells transfected with the EGFR alone indicates that the precipitation of Grb2 was the result of an interaction between the *cbl* protein and Grb2. Using a Gst-Grb2 fusion protein to precipitate *in vitro* translated deletion mutants of *cbl*-b we have localized the binding of Grb2 to the proline rich C-terminus half of the *cbl*-b protein (data not shown). The N-terminus of c-*cbl* contains a unique PTB which has been shown to directly bind to tyrosine phosphorylated proteins (Lupher *et al*., 1996, 1997). A construct of *cbl*-b containing only the first 349 amino acids (including the conserved PTB) was able to bind to the activated EGFR but was unable to interact with the Gst-Grb2 fusion protein (data not shown). Thus, like c-*cbl*, *cbl*-b is able to bind to the EGFR through both an adaptor mediated and PTB domain mediated mechanism.

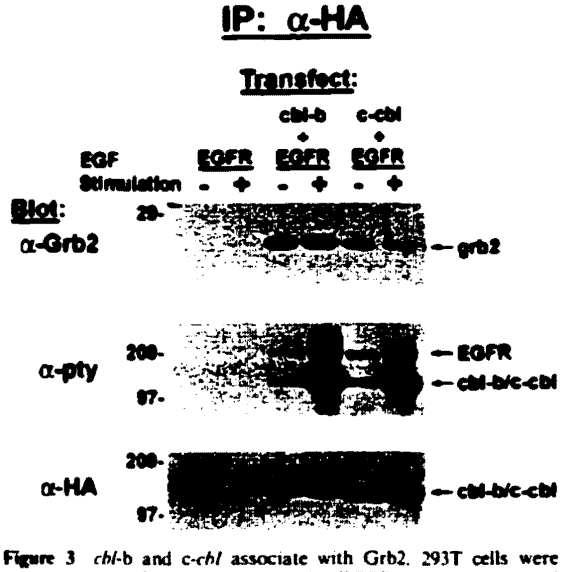


Figure 3 *cbl*-b and c-*cbl* associate with Grb2. 293T cells were transiently transfected with either the EGFR alone, HA tagged *cbl*-b and the EGFR, or HA tagged c-*cbl* and the EGFR. The cells were starved for 4 h, incubated with or without EGF (100 ng/ml) for 10 min and then the cells were lysed. *cbl*-b and c-*cbl* were immunoprecipitated using the z-HA antibody. The precipitated proteins were run on parallel gels, transferred, and immunoblotted with the antibodies shown to the left of the blots. The position of each protein is indicated to the right. Each protein (EGFR, *cbl*-b or c-*cbl* and Grb2) was equally expressed in lysates (data not shown). Molecular weight standards (in kDa) are shown to the left of the blots

Since both *cbl*-2 and c-*cbl* are phosphorylated and recruited to the EGFR upon stimulation and interact with the receptor through similar mechanisms, we investigated whether the two proteins compete for their interaction with the EGFR. 293T cells were transfected with c-*cbl* and the EGFR in the presence or absence of an excess of *cbl*-b (Figure 4a) or with *cbl*-b and the EGFR in the presence or absence of an excess of c-*cbl* (Figure 4b) and then the cells were starved and then stimulated with EGF. Lysates from each transfection were immunoprecipitated with the anti-EGFR antibody. Less c-*cbl* co-precipitates with the EGFR in the presence of excess *cbl*-b than in the absence of *cbl*-b (Figure 4a) and similarly, less *cbl*-b co-precipitates with the EGFR in the presence of excess c-*cbl* than in the absence of c-*cbl* (Figure 4b). The excess of competing *cbl* protein was demonstrated by probing the lysates with the anti-HA antibody which detects both proteins (Figure 4, lysate panels). These data suggest that *cbl*-b and c-*cbl* compete for binding to the EGFR.

Interestingly, when lysates containing both *cbl* proteins were probed with anti-pty the level of EGF induced phosphorylation of the 120 kDa protein was not significantly increased compared to cells expressing only one or the other of the *cbl* proteins (Figure 4a and b, lysate panels). Immunoprecipitation of c-*cbl* from the lysates in Figure 4a or *cbl*-b from the lysates in Figure 4b revealed that in the presence of excess of the other *cbl* protein, phosphorylation of the precipitated protein was decreased (data not shown). This suggests that the *cbl* proteins are phosphorylated by the same kinases and compete with one another as substrates.

Effects of cbl-*b and* c-cbl *on EGF induced cell growth of 32D/EGFR cells*

In order to investigate the functional role of the interaction of the *cbl* proteins with the EGFR, we transfected *cbl*-b or c-*cbl* into 32D cells which overexpress the EGFR. The 32D cell line is a murine hematopoietic cell line which is absolutely dependent on exogenous IL-3 for sustained growth and it rapidly undergoes apoptosis in the absence of IL-3 (Greenberger *et al*., 1983; Ihle *et al*., 1981, 1982; Prystowsky *et al*., 1982). 32D cells do not normally express any endogenous EGFR (or other members of the EGFR family) and do not grow in EGF (Alimandi *et al*., 1997). In contrast, 32D cells overexpressing the EGFR (32D/EGFR) can be grown in the presence of either IL-3 or EGF (Pierce *et al*., 1988). These 32D/EGFR cells, used in our experiments, were maintained in

growth media supplemented with 5% conditioned medium containing IL-3 (which will be referred to as IL-3). 32D/EGFR cells were transfected with either *cbl*-b, c-*cbl*, or a vector control and stable clones were selected which were able to grow in medium containing IL-3 and G418. These clones were then analysed for

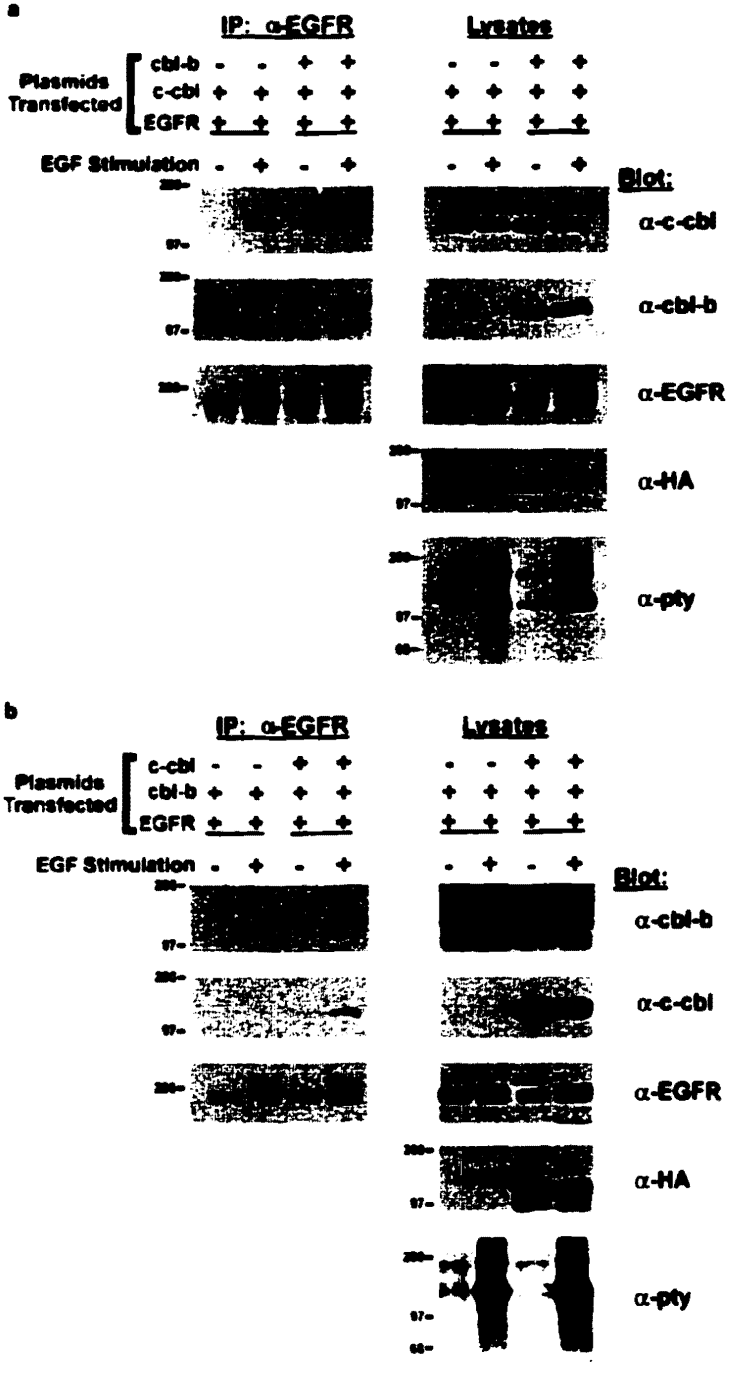
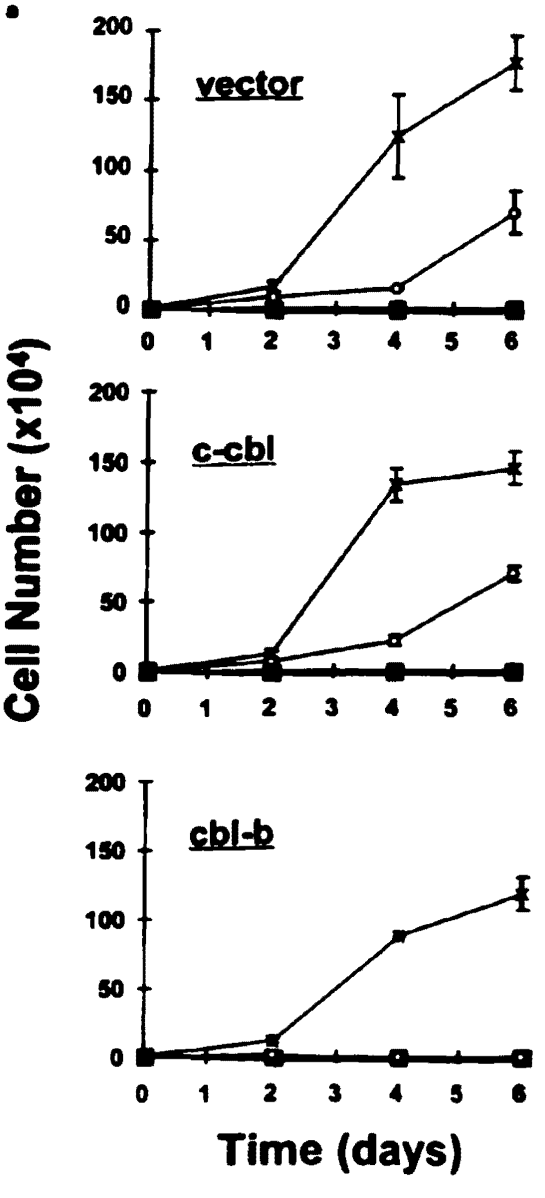


Figure 4 *cbl*-b competes with c-*cbl* for binding to the EGFR. (**a**) 293T cells were transiently transfected with c-*cbl* (0.5 μg) and the EGFR (1 μg) in the presence or absence of an excess of *cbl*-b (5 μg). (**b**) 293T cells were transiently transfected with *cbl*-b (0.5 μg) and the EGFR (1 μg) in the presence or absence of an excess of c-*cbl* (5 μg). The cells were starved for 4 h, incubated with or without EGF (100 ng/ml) for 10 min and then cell lysates were prepared. In the left panels of each figure, the EGFR was immunoprecipitated and then the precipitated proteins were run on parallel gels, transferred, and immunoblotted with the antibodies shown to the right of the blots. The top three right panels in each figure show the expression of c-*cbl*, *cbl*-b and the EGFR in the cell lysates used for the immunoprecipitation. The total level of c-*cbl* + *cbl*-b in the lysates is shown in the anti-HA probed panel on the right of each figure. The bottom right panel in each figure shows the phosphorylation induced by EGF in the lysates. The plasmids transfected are indicated along the top of the figure

their ability to grow in EGF or IL-3. 32D/EGFR cells overexpressing *cbl*-b showed markedly inhibited growth in EGF compared to c-*cbl* transfectants and vector controls (Figure 5a and b), while only slight inhibition of growth of *cbl*-b clones was observed in IL-3. In contrast, both c-*cbl* and vector clones grew well in either EGF or IL-3. Even at lower concentrations of IL-3, which only stimulated growth to the same degree as EGF, there was no significant difference between the clones expressing *cbl*-b and those overexpressing c-*cbl* or the vector controls. Thus the inhibition is specific for the EGFR pathway. The expression of *cbl*-b, c-*cbl* and the EGFR in representative clones is shown in Figure 5c. Both *cbl*-b and c-*cbl* were expressed at levels five- to tenfold above that of the endogenous protein. Reprobing the blots with the anti-HA antibody demonstrated that *cbl*-b and c-*cbl* were expressed at similar levels (data not shown). There was no significant difference between the clones in the total expression (Figure 5c) or in the cell surface expression of the EGFR protein determined by flow cytometry (data not shown).

Cells overexpressing *cbl*-b cultured in EGF were shrunken, refractile cells with pyknotic nuclei and were similar to those seen when cultured in the absence of growth factor (Figure 6a). TUNEL assays, which detect DNA breaks characteristic of apoptosis *in situ* (Ben-Sasson *et al*., 1995), revealed that the *cbl*-b overexpressing cells cultured in the absence of growth factor and those cultured in EGF were undergoing apoptosis (Figure 6a, top panels). In contrast, the *cbl*-b clones grew well in IL-3 with little or no evidence of



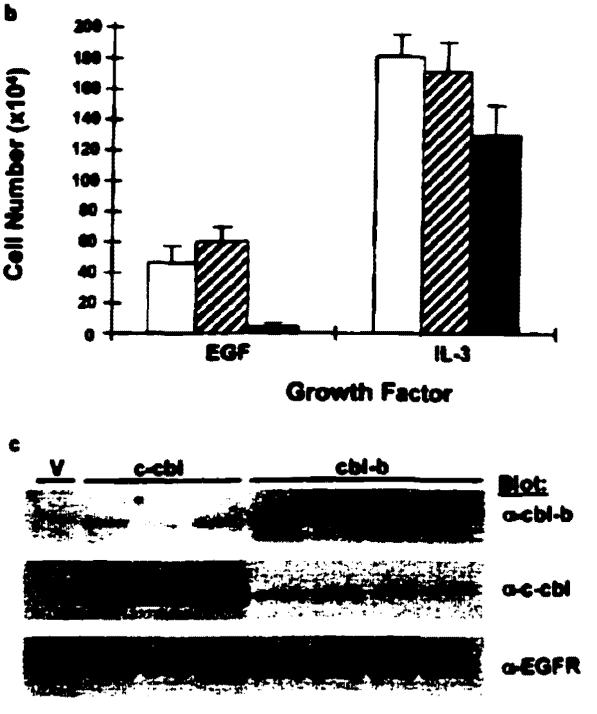
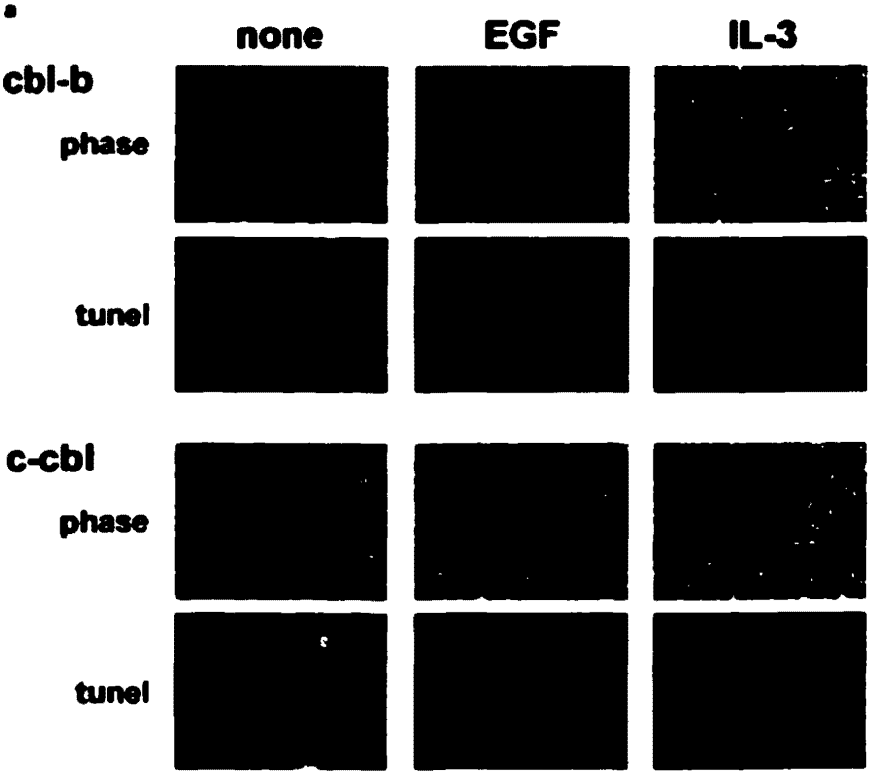


Figure 5 *cbl*-b, but not c-*cbl*, inhibits EGF stimulated growth of 32D/EGFR cells. (a) Stable clones of 32D/EGFR cells expressing either *cbl*-b, c-*cbl*, or a control vector were plated in triplicate wells at 10^4 cells and grown in the absence of growth factor (closed squares), in EGF (open circles), or in IL-3 (-x-). The number of live cells was counted after 2, 4 and 6 days in culture using trypan blue to assess viability. The numbers represent the average ±s.d. The live cell number is shown on the Y axis and the time in culture is shown along the X axis. (b) Composite growth data for stable clones. Independent stable clones of 32D EGFR cells expressing either *cbl*-b (black bars; $n = 8$), c-*cbl* (striped bars; $n = 7$), or a control vector (open bars; $n = 6$) were plated in triplicate wells at 10^4 cells and grown in EGF or in IL-3. The number of live cells was counted after 6 days in culture using Trypan blue to assess viability. The numbers represent the average ± the s.e.m. for multiple independent clones. The live cell number is shown on the Y axis and the growth factor added is shown along the X axis. Cells from all clones died within 1 day when plated in the absence of growth factors. (c) Expression of *cbl*-b, c-*cbl* and the EGFR in representative stable clones of 32D EGFR. Cell lysates were separated by SDS PAGE on parallel gels, transferred and immunoblotted with the antibodies shown to the right of the blots

apoptosis. Cells overexpressing c-*cbl* underwent apoptosis when grown in the absence of growth factor but grew well in either EGF or IL-3 with little or no evidence of apoptosis when grown in either growth factor (Figure 6b, bottom panels). The vector control cells behaved like the cells overexpressing c-*cbl* (data not shown). In order to quantitate the fraction of cells undergoing apoptosis, cells were stained with propidium iodide and the fraction of cells in the sub-G1 peak was determined. A high proportion of cells in the *cbl*-b clones cultured in EGF were undergoing apoptosis when compared to those grown in IL-3 (Figure 6b). In contrast, only a small fraction of the cells in the c-*cbl* clones and vector controls undergoes apoptosis when grown in the presence of either growth factor (Figure 6b). The proportion of apoptotic cells in the *cbl*-b clones grown in EGF increased with time in culture but this increase was not as rapid as seen in cells grown in the absence of growth factor (Table 1).

Cell cycle analysis revealed that the large increase in apoptotic cells in *cbl*-b clones grown in EGF was the only clear difference between *cbl*-b clones and the c-*cbl*



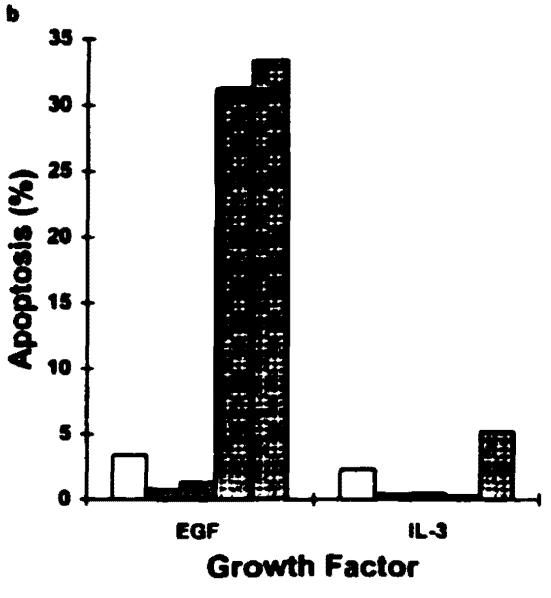


Figure 6 32D/EGFR cells expressing *cbl*-b undergo apoptosis in EGF. (**a**) Stable clones of 32D EGFR cells expressing either *cbl*-b or c-*cbl* were plated at 10^4 cells/plate and grown in the absence of growth factor (none), in EGF, or in IL-3. The cells were grown for 3 days and then TUNEL assays to demonstrate apoptosis were performed. Cell cytospins showing phase (phase) and fluorescence (tunel) photomicrographs of the same field for each sample are shown at 1000 × magnification (**b**) Stable clones of 32D EGFR expressing *cbl*-b (gray bars), c-*cbl* (striped bars), or a control vector (open bars) were cultured as above for 5 days. The percentage of apoptotic cells were determined from the number of cells in the sub-G1 population when analysed by propidium iodide staining and flow cytometry. Results are shown for two independent stable 32D EGFR clones expressing *cbl*-b, two clones expressing c-*cbl* and one vector control

Table 1 Apoptosis of 32D/EGFR cells overexpressing *cbl*-b

Growth factor	Apoptosis (%) day 1	day 3	day 5
none	58	46	53
EGF	6	20	33
IL-3	0.4	0.6	5

clones and vector controls. There was no evidence of cell cycle arrest in the *cbl*-b clones cultured in EGF at early (within the first 16 h in culture) or late (after several days in culture) time points. In contrast, cells cultured in the absence of growth factor showed clear evidence of G1 arrest within the first 16 h in culture. Consistent with the lack of cell cycle arrest in the *cbl*-b clones cultured in EGF, thymidine incorporation by the *cbl*-b clones cultured in EGF for 1–2 days was similar to that of c-*cbl* clones and vector controls (data not shown).

Both *cbl*-b and c-*cbl* were phosphorylated and recruited to the EGFR in the 32D/EGFR clones overexpressing these proteins (data not shown). In order to investigate the effects of *cbl*-b and c-*cbl* on EGFR signaling, we measured the EGF induced activation of MAP kinase (MAPK) and Jun kinase (JNK) in clones overexpressing *cbl*-b, c-*cbl*, or the vector control (Figure 7a and b). EGF stimulation of the 32D/EGFR cells overexpressing *cbl*-b induced rapid stimulation (within 5 min) of both MAPK and JNK activities and this stimulation was similar to that seen in c-*cbl* overexpressing cells or the vector controls. However, the MAPK and JNK activities in the *cbl*-b clones returned towards the baseline earlier (e.g. 30 min) than the activities in the c-*cbl* and vector controls.

The activation of the serine threonine kinase AKT by growth factor receptors has been shown to inhibit apoptosis (Franke *et al*., 1997). This activation results from the specific phosphorylation of AKT on serine 473 and threonine 308 in response to growth factor stimulation (Alessi *et al*., 1996). Because of the marked increase in apoptosis we observed in *cbl*-b clones cultured in EGF, we assayed the activation of AKT in clones overexpressing *cbl*-b, c-*cbl*, or the vector controls (Figure 8). The EGF induced activation of AKT was markedly decreased in the clones overexpressing *cbl*-b compared to cells overexpressing c-*cbl* or the vector controls. In addition, as seen with MAPK and JNK above, the duration of the activation of AKT was shortened in cells overexpressing *cbl*-b compared to cells overexpressing c-*cbl* or the vector controls.

AKT is activated downstream of phosphatidylinositol 3-kinase (PI 3-kinase) (Franke *et al*., 1997) and c-*cbl* has been shown to associate with the 85 kDa regulatory subunit (p85) of PI 3-kinase upon activation of the EGFR (Miyake *et al*., 1997; Smit and Borst, 1997). To test whether *cbl*-b interacts with the 85 kDa subunit of PI 3-kinase, 293T cells were transfected with the EGFR or *cbl*-b and the EGFR. The *cbl*-b protein was immunoprecipitated with the anti-HA antibody and the precipitates were probed for the p85 subunit of PI 3-kinase (Figure 9). The p85 subunit of PI 3-kinase co-precipitated with the heavily phosphorylated *cbl*-b from the EGF stimulated lysates. The lack of the p85 subunit of PI 3-kinase in the precipitates from the cells

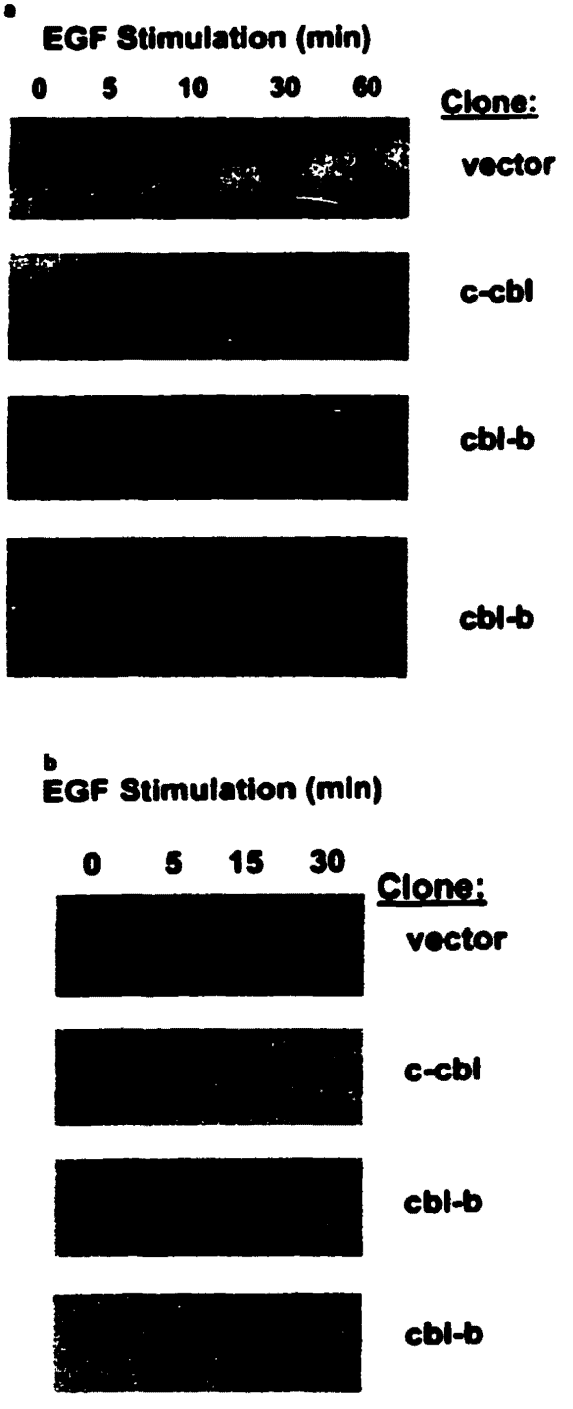


Figure 7 Activation of MAPK and JNK is not sustained in 32D EGFR cells overexpressing *cbl*-b. EGF stimulation of MAPK (**a**) and JNK (**b**) was assayed in 32D EGFR cells expressing *cbl*-b (two independent clones), c-*cbl*, or a vector control (as described in the Materials and methods). The cells were starved and then stimulated for the indicated times with EGF (100 ng/ml). Cells were lysed, MAPK or JNK was immunoprecipitated, and activity was assayed by phosphorylation of myelin basic protein for MAPK or phosphorylation of GST-Jun for JNK. The phosphorylation of the substrate was assessed by SDS PAGE and autoradiography. Immunoblot analysis of one fifth of the precipitated proteins (either MAPK or JNK) demonstrated that equal amounts of protein were used in the kinase assays (data not shown)

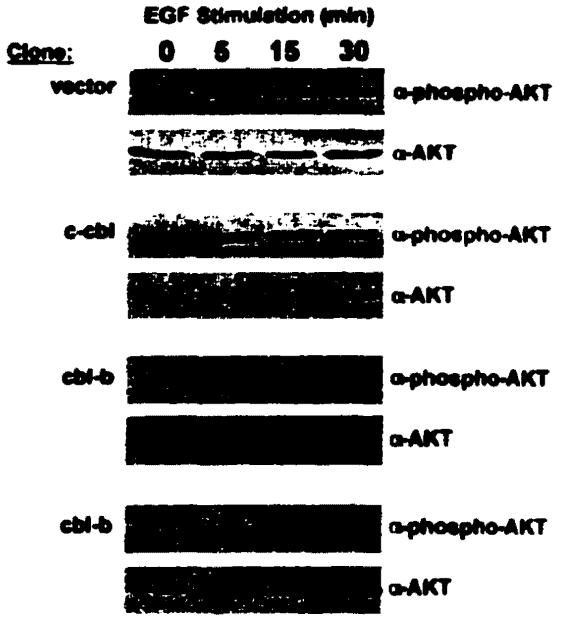


Figure 8 AKT activation is inhibited in 32D/EGFR cells overexpressing *cbl*-b. EGF stimulation of AKT activation was assayed in 32D/EGFR cells expressing *cbl*-b (two independent clones), c-*cbl*, or a vector control. The cells were harvested and then stimulated for the indicated times with EGF (100 ng/ml). Cells were lysed and AKT activation was assayed by immunoblotting with an antibody specific for activated phosphorylated AKT (α-phospho-AKT). Total AKT was assessed by probing a parallel gel with antibody that recognizes both active and inactive AKT (α-AKT)

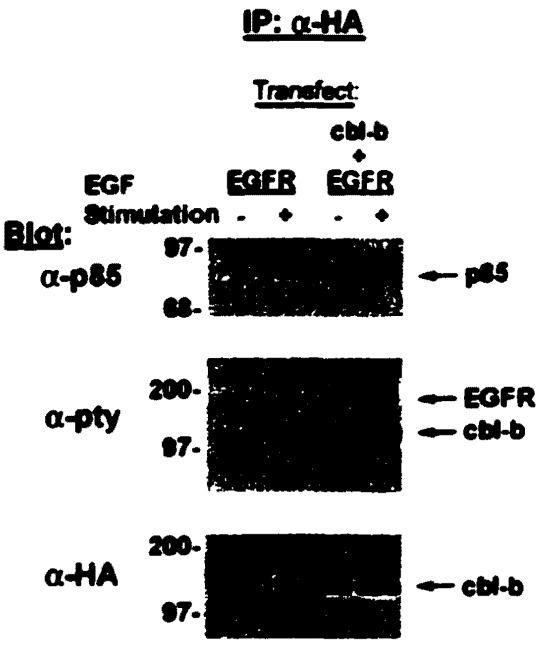


Figure 9 *cbl*-b associates with the p85 subunit of PI-3K. 293T cells were transiently transfected with either the EGFR alone or HA tagged *cbl*-b and the EGFR. The cells were starved for 4 h, incubated with or without EGF (100 ng/ml) for 10 min, and then the cells were lysed. *cbl*-b was immunoprecipitated using the α-HA antibody. The precipitated proteins were run on parallel gels, transferred and immunoblotted with the antibodies shown to the left of the blots. The position of each protein is indicated to the right. Each protein (EGFR, *cbl*-b and p85) was equally expressed in lysates (data not shown). Molecular weight standards (in kDa) are shown to the left of the blots

transfected with the EGFR alone indicates that the precipitation of p85 was the result of an interaction between the *cbl*-b protein and p85 (Figure 9, bottom panel). We were unable, however, to demonstrate any EGF induced association between either *cbl*-b or c-*cbl* and the p85 subunit of PI 3-kinase in the 32D/EGFR clones overexpressing *cbl*-b or c-*cbl* respectively (data not shown).

Discussion

The *cbl* family of proteins is found in metazoans from nematodes to vertebrates and the proteins have several highly conserved domains including a novel N-terminal PTB motif and a zinc finger (Blake *et al*., 1991; Hime *et al*., 1997; Keane *et al*., 1995; Lupher *et al*., 1996, 1997; Meisner *et al*., 1997; Yoon *et al*., 1995). A role for the *cbl* proteins in EGFR signaling was first demonstrated in *C. elegans* by genetic studies that show that sli-1 (the *cbl* homolog) is a negative regulator of the Let-23 receptor tyrosine kinase (the EGFR homolog) in vulva development (Jongeward *et al*., 1995; Yoon *et al*., 1995). These developmental effects have been extended to *Drosophila* where the *cbl* homolog has been shown to associate with the *Drosophila* EGFR and overexpression of *Drosophila cbl* in the eye of *Drosophila* embryos inhibits EGFR dependent photoreceptor cell development (Hime *et al*., 1997; Meisner *et al*., 1997). In mammalian cells, several studies have shown that c-*cbl* becomes phosphorylated and recruited to the EGFR upon stimulation (Bowtell and Langdon, 1995; Fukazawa *et al*., 1996; Galisteo *et al*., 1995; Khwaja *et al*, 1996; Levkowitz *et al*., 1996; Meisner and Czech, 1995; Odai *et al*., 1995a; Soltoff and Cantley, 1996; Tanaka *et al*., 1995; Ueno *et al*., 1997). However, biological consequences of this interaction have not been demonstrated.

The data presented here show that *cbl*-b, but not c-*cbl*, inhibits EGFR induced growth in 32D/EGFR cells (Figure 5). These cells are absolutely dependent on growth factor (either EGF or IL-3) and rapidly undergo apoptosis in the absence of exogenous growth factor (Greenberger *et al*., 1983; Ihle *et al*., 1981, 1982; Pierce *et al*., 1988; Prystowsky *et al*., 1982). The inhibition of growth in the *cbl*-b clones is not associated with any cell cycle arrest but it is associated with a dramatic increase in the fraction of cells undergoing apoptosis (Figure 6). Recent findings have established that cell growth in response to growth factor stimulation requires active inhibition of apoptosis via the activation of AKT in addition to stimulation of cell cycle progression (Ahmed *et al*., 1997; Dudek *et al*., 1997; Franke *et al*., 1995, 1997; Hemmings, 1997; Kulik *et al*., 1997). EGF stimulated cells overexpressing *cbl*-b had a markedly decreased activation of AKT and the duration of AKT activation was foreshortened compared to cells overexpressing c-*cbl* or the vector controls (Figure 8). This result is consistent with the increased proportion of cells undergoing apoptosis.

The inhibition of EGFR stimulated growth in the 32D/EGFR cells by *cbl*-b and not by c-*cbl* clearly

demonstrates distinct biological roles for *cbl*-b and c-*cbl*. The precise molecular mechanism by which *cbl*-b exerts its inhibitory effect is not yet known. In both transiently transfected cells (293T cells) and in stable clones (32D/EGFR cells), *cbl*-b and c-*cbl* are phosphorylated and recruited to the EGFR upon activation of the receptor. Both *cbl* proteins interacted with the Grb2 adaptor protein in the unstimulated cells and we saw a slight increase in the association between *cbl*-b and Grb2 upon stimulation but not between c-*cbl* and Grb2 (Figure 3). While some investigators have shown modest increases in the interaction of c-*cbl* with Grb2 upon EGF stimulation (Fukazawa *et al*., 1996; Khwaja *et al*., 1996; Meisner and Czech, 1995), others have found a constitutive interaction between c-*cbl* and Grb2 (Levkowitz *et al*., 1996; Odai *et al*., 1995b). The binding of the proline rich C-terminus of *in vitro* translated *cbl*-b to a Gst-Grb2 fusion protein is consistent with an SH3 mediated interaction. We also have found that the N-terminus of *cbl*-b, like the N-terminus of c-*cbl* (Miyake *et al*., 1997; Smit and Borst, 1997), is able to associated with the EGFR (not shown). The similarities of *cbl*-b and c-*cbl* in binding to the EGFR and Grb2 (Figures 2 and 3), and the ability of each *cbl* protein to decrease the binding of the other to the EGFR (Figure 4) suggest that both interact with the receptor through a common site and/or mechanism. The binding site of the *cbl* proteins on the EGFR has not yet been identified. There was no inhibition of EGF induced phosphorylation of the EGFR (Figure 1) suggesting that binding of *cbl*-b (or c-*cbl*) to the EGFR does not directly affect its activation by EGF. This is consistent with prior observations that c-*cbl* does not directly alter the phosphorylation or kinase activity of the EGFR (Thien and Langdon, 1997). However, there are some published data which suggest that c-*cbl* can inhibit phosphorylation of the EGFR (Ueno *et al*., 1997) and the reason for these differing results is unknown. Cotransfection of the *cbl* proteins did decrease the phosphorylation of the unstimulated EGFR when the EGFR was overexpressed in 293T cells indicating that the *cbl* proteins may indeed inhibit activation of the EGFR. No such high levels of phosphorylated unstimulated EGFR were seen in the vector controls or parental cell line of the 32D/EGFR cells. This suggests that the high level of phosphorylated unstimulated EGFR may be unique to 293T cells or a consequence of the high levels of expression of the EGFR obtained upon transient transfection. The significance of the decrease in phosphorylation of the unstimulated EGFR by *cbl*-b and c-*cbl* in the 293T cells remains to be determined.

Overexpression of *cbl*-b inhibited activation of downstream MAPK, JNK and AKT pathways (Figures 7 and 8). *cbl*-b has been shown to inhibit JNK activation by Vav (Bustelo *et al*., 1997) but there are no published data addressing the effects of *cbl*-b on MAPK or AKT. Previous reports do not show inhibition of MAPK activation by c-*cbl* in NIH3T3 cells (Bowtell and Langdon, 1995; Thien and Langdon, 1997; Ueno *et al*., 1997). AKT activation was more profoundly affected than MAPK activation and this is consistent with the increase in apoptosis and absence of cell cycle arrest. AKT activation is downstream of growth factor induced activation of PI 3-kinase (Franke *et al*., 1997). c-*cbl* has been shown to associate with the 85 kDa regulatory subunit of PI 3-kinase upon activation of a variety of receptors, including the EGFR (Miyake *et al*., 1997; Smit and Borst, 1997) and c-*cbl* has been demonstrated to enhance IL-4 induced PI 3-kinase activity and mitogenic and survival signals in Ba/F3 cells (Ueno *et al*., 1998). We have demonstrated an EGF induced association between *cbl*-b and the p85 regulatory subunit of PI 3-kinase in transiently transfected 293T cells that overexpress *cbl*-b (Figure 9). *cbl*-b has one consensus binding site for the SH2 domain of PI 3-kinase (Y_{863}CEM) which is conserved between all of the *cbl* family of proteins. However, *cbl*-b lacks the second binding site found in c-*cbl* (Y_{731} EAM) which is believed to be the site at which PI 3-kinase binds to c-*cbl* (Liu *et al*., 1997). We were unable to demonstrate any interaction of either *cbl*-b or c-*cbl* with the p85 subunit of PI 3-kinase in 32D/EGFR cells. We were also unable to demonstrate any direct effect of *cbl*-b on the binding of c-*cbl* to p85 in the transiently transfected 293T cells (unpublished observation). Thus, the mechanism by which *cbl*-b inhibits AKT activation in the transfected 32D/EGFR cells remains to be determined. The recruitment of *cbl*-b to the EGFR upon activation and the inhibition of activation of multiple downstream kinases suggests that *cbl*-b functions at a step in the pathway close to the receptor. The foreshortening of MAPK, JNK and AKT activation by *cbl*-b suggests that *cbl*-b may enhance feedback inhibition of the EGFR.

Overall, our data demonstrate that a mammalian *cbl* protein, *cbl*-b is able to inhibit EGFR-induced growth and this inhibition is due to a failure to activate anti-apoptotic pathways. These data further demonstrate that while these proteins share some structural and biochemical similarities, there are major functional differences between the *cbl*-b and c-*cbl* proteins.

Materials and methods

Antibodies

Rabbit polyclonal anti-*cbl*-b (H121; Santa Cruz Biotechnology) and anti-c-*cbl* (C-15; Santa Cruz Biotechnology) were used for both immunoblotting and immunoprecipitation. Mouse monoclonal anti-HA (12CA5; Boehringer Mannheim) and anti-EGFR (Ab3; Oncogene Science) were used for immunoprecipitation and rabbit polyclonal anti-HA antibody (Y-11, Santa Cruz Biotechnology), anti-EGFR (1005, Santa Cruz Biotechnology), anti-p85 subunit of PI 3-kinase (06-195; Upstate Biotechnology), and anti-Grb2 (C-23; Santa Cruz Biotechnology) were used for immunoblotting. Horseradish peroxidase linked anti-phosphotyrosine (4G10; Upstate Biotechnology Inc.) was used for immunoblotting. Horseradish peroxidase linked donkey anti-rabbit Ig (Amersham) was used along with ECL detection reagent (Super Signal; Pierce) to visualize immunoblots. Anti-ERK antibody (SC-154, Santa Cruz Biotechnology) was used to immunoprecipitate active ERK1 and ERK2 for the immunocomplex MAPK assay.

Plasmids

A nine amino acid epitope tag from the influenza virus hemagglutin protein (HA) (Wilson *et al*., 1984) was added to the C-terminal of the full length human *cbl*-b open reading frame (Keane *et al*., 1995) by PCR and the cDNA

was cloned into pCEFL, a mammalian expression vector with the elongation factor promoter and a neomycin selectable marker (provided by Dr Silvio Gutkind). The construct was sequenced to verify that there were no mutations introduced. HA-tagged c-*cbl* was provided by Dr Wallace Langdon (Andoniou *et al.*, 1996). This construct was also cloned into the pCEFL vector. The GST-cjun79 fusion construct used as a substrate in the JNK assay was provided by Dr Silvio Gutkind. The fusion protein was purified from bacterial lysates as previously described (Coso *et al.*, 1995).

Immunoblotting and immunoprecipitation

Immunoblotting was performed as previously described (Ausubel *et al.*, 1994) and detection by chemiluminescence was performed using ECL (Amersham) according to the instructions provided. Briefly, for immunoprecipitation protein from total cell lysate was incubated for 30 min on ice with antibody. Immune complexes were recovered by incubation with protein A/G+ agarose beads (Santa Cruz Biotechnology) at 4°C for 1 h with tumbling. Immune complexes were washed five times in cold lysis buffer, resuspended in 2× loading buffer (Promega), boiled for 5 min, and then resolved by 10% SDS-PAGE. The gels were transferred to nitrocellulose membranes (Schleicher and Schuell) or to PVDF membranes (Immobilon P, Millipore).

Cell culture

293 cells transfected with the SV40 large T antigen (293T) (provided by Mike Erdos) were maintained in culture in DMEM supplemented with 10% fetal calf serum and 1% Penicillin-Streptomycin (Pen-Strep) and were transfected with various constructs using calcium phosphate (5 Prime→3 Prime, Inc.) according to the protocol included with the reagents. To measure the effects of EGF stimulation, 293T cells were grown to 70% confluence and serum starved in DMEM supplemented with 0.1% bovine serum albumin (BSA) and 1% Pen-Strep for 4 h. One hundred ng/ml of recombinant human EGF (Collaborative Biomedical Products) was added for the times indicated, the cells were washed two times in ice-cold PBS containing 0.2 mM sodium orthovanadate and the cells were lysed in ice-cold lysis buffer (10 mM Tris HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Triton X 100, 10% Glycerol, 1 mM 4-(2 aminoethyl) benzenesulfonyl fluoride (AEBSF), 20 μg/ml Leupeptin, 20 μg/ml Aprotinin, 10 μg/ml Pepstatin, 2 mM sodium orthovanadate). The lysates were cleared of debris by centrifugation at 16 000 *g* for 15 min at 4°C.

32D/EGFR cells were maintained in culture in RPMI 1640 supplemented with 15% fetal calf serum, 5% WEHI 3B conditioned medium and 1% Pen-Strep. The WEHI 3B cell line was maintained in RPMI 1640 supplemented with 15% fetal calf serum and 1% Pen-Strep. WEHI 3B conditioned media containing IL-3 was produced by culturing the WEHI 3B cells to a high density and then harvesting the supernatants. 32D/EGFR cells were transfected by electroporation as previously described (Pierce *et al.*, 1988) and stable clones were selected and maintained in growth media supplemented with 750 μg/ml G418 (GIBCO-BRL). To assess the growth in different conditions, cells were pelleted, resuspended in RPMI 1640 containing 15% FCS, 1% Pen-Strep, but no growth factor. Cells were seeded in 24 well plates at 1×10^4 cells/well. Either EGF (10 ng/ml), IL-3 (5% conditioned media) or no growth factor was added. The cells were incubated for the indicated time and then cells were harvested and counted using trypan blue to assess viability. Each data point was done in triplicate.

TUNEL assays to detect fragmented DNA *in situ* (Ben-Sasson *et al.*, 1995) were peformed on cell cytospins using the *In Situ* Death Detection Kit (Boehringer Mannheim).

Kinase assays

MAPK was assayed as previously described (Crespo *et al.*, 1994). Briefly, cells were stimulated with EGF (100 ng/ml) for the indicated time, washed in ice-cold PBS containing 0.2 mM sodium orthovanadate, and the cells were lysed in ice-cold lysis buffer (20 mM HEPES, pH 7.5, 2.5 mM $MgCl_2$, 10 mM EGTA, 40 mM β-glycerophosphate, 1.0% Nonidet P-40, 1 mM DTT, 1 mM AEBSF, 20 μg/ml Leupeptin, 20 μg/ml Aprotinin, 2 mM sodium orthovanadate). Cleared lysates were incubated at 4°C with tumbling with anti-Erk antibody and protein A/G+ agarose beads for 1 h. The beads were washed three times with PBS containing 1% Nonidet P-40 and 2 mM sodium orthovanadate, once with 100 mM Tris (pH 7.5) containing 0.5 M LiCl, and once with kinase reaction buffer (12.5 mM MOPS, pH 7.5, 7.5 mM $MgCl_2$, 3.3 μM DTT, 12.5 mM β-glycerophosphate, 0.5 mM EGTA, 0.5 mM NaF, 0.5 mM sodium orthovanadate). The beads were resuspended in 30 μl of kinase reaction buffer containing 10 μCi [γ-^{32}P]ATP (3000 Ci/mmol; Amersham), 20 μM cold ATP and 1.5 mg/ml Myelin Basic Procin (MBP) as a substrate. The reactions were incubated at 30°C for 20 min and terminated by the addition of 15 μl of 4× Laemmli buffer. The samples were heated to 95°C for 5 min and analysed by SDS-PAGE on 12% gels. The gels were dried and the phosphorylated MBP was assessed by autoradiography using AR film and an intensifying screen (Kodak).

JNK was assayed as previously described (Coso *et al.*, 1995; Crespo *et al.*, 1994). Briefly, cells were stimulated with EGF (100 ng/ml) for the indicated time, washed in ice-cold PBS containing 0.2 mM sodium orthovanadate and the cells were lysed in ice-cold lysis buffer (25 mM HEPES, pH 7.5, 300 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 20 mM β-glycerophosphate, 0.1% Triton X 100, 1 mM AEBSF, 20 μg/ml Leupeptin, 0.1 mM sodium orthovanadate). Cleared lysates were incubated at 4°C with tumbling with 1 μg of GST-cjun79 protein bound to glutathione-agarose beads for 3-4 h. The beads were washed three times with PBS containing 1% Nonidet P-40 and 1 mM sodium orthovanadate, once with 100 mM Tris (pH 7.5) containing 0.5 M LiCl, and once with kinase reaction buffer (25 mM HEPES pH 7.5, 20 mM $MgCl_2$, 2 mM DTT, 20 mM β-glycerophosphate, 0.1 mM sodium orthovanadate). The beads were resuspended in 30 μl of kinase reaction buffer containing 1 μCi [γ-^{32}P]ATP (3000 Ci/mmol; Amersham) and 50 μM cold ATP, incubated at 30°C for 20 min, and the reactions were terminated by the addition of 15 μl of 4× Laemmli buffer. The samples were heated to 95 C for 5 min and analysed by SDS-PAGE on 12% gels. The gels were dried and the phosphorylated GST-cjun79 was assessed by autoradiography using Biomax MR film (Kodak).

AKT activation was assessed by the specific phosphorylation of AKT on serine 473 using the Phosphoplus AKT (Ser473) antibody kit (New England Biolabs) according to the method provided with the kit.

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Paper 2

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cbl-b Inhibits EGF-Receptor-Induced Apoptosis by Enhancing Ubiquitination and Degradation of Activated Receptors

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Studies in *C. elegans* and *Drosophila melanogaster* suggest that cbl proteins are inhibitors of epidermal growth factor receptor (EGFR) function. Here we describe that overexpression of cbl-b, a homologue of the c-cbl protooncogene, inhibits EGFR-induced apoptosis in MDA-MB-468 breast cancer cells. Overexpression of cbl-b results in a shortened duration of EGFR activation upon EGF stimulation. This is demonstrated by decreased amounts of phosphorylated EGFR as well as by inhibition of multiple downstream signaling pathways. The inhibition of signaling by cbl-b results from increased ubiquitination and degradation of the activated EGFR. The inhibitory effects of cbl-b overexpression on apoptosis and on EGFR signaling are reversed by blocking proteosomal degradation of the EGFR. These data demonstrate that the mechanism by which cbl-b inhibits EGFR-induced apoptosis is by activation-dependent degradation of the EGFR. They imply that this mechanism may be a general one whereby cbl proteins regulate intracellular signaling.

The cbl proteins are a highly conserved family of proteins found in metazoans from nematodes to vertebrates and these proteins have several highly conserved domains including a novel N-terminal SH2 domain and a RING finger (1–8). There are three mammalian cbl proteins: c-cbl, cbl-b, and the recently described cbl-3 (1, 3, 4). cbl-b is structurally most similar to c-cbl while cbl-3 appears to be more closely related to the shorter *C. elegans* and *Drosophila* cbl proteins (3, 4). The cbl proteins are tyrosine phosphorylated upon activation of a variety of growth factor receptors and they associate with many proteins containing Src Homology 2 and 3 domains (reviewed in (9, 10)). These diverse interactions modulate signaling through many pathways (9, 10). For example, in mast cells overexpression of c-cbl inhibited FcεRI induced activation of Syk kinase and downstream serotonin release (11) and overexpression of cbl-b in Cos cells inhibited Vav-induced Jun Kinase activation (12). Recent work has shown that c-cbl-deficient mice have hyperplastic hematopoietic and breast tissue consistent with a negative regulatory role in cellular proliferation for c-cbl (13). Together these evolutionary and biochemical data indicate that the cbl proteins are important regulators of intracellular signaling and consequently of cell function and development.

Genetic studies in *C. elegans* first demonstrated that cbl proteins are regulators of epidermal growth factor receptor (EGFR) function. sli-1 (the *C. elegans* cbl homologue) is a negative regulator of the Let-23 receptor tyrosine kinase (the EGFR homologue) in vulva development (6, 14). Developmental effects have also been demonstrated in *Drosophila* where D-cbl has been shown to associate with the *Drosophila* EGFR and overexpression of D-cbl in the eye of *Drosophila* embryos inhibits EGFR dependent photoreceptor cell development (2, 5). In mammalian cells, several studies have shown that c-cbl becomes phosphorylated and recruited to the EGFR upon stimulation and alters signaling (reviewed in (10, 15)). We previously reported that cbl-b inhibits EGF induced growth and EGFR signaling but the mechanism of this inhibition was not clear. Recent findings with c-cbl have implicated ubiquitination and degradation of growth factor receptors as a possible mechanism of this inhibition (16–19).

To study the function of cbl-b in regulating EGFR function further, we investigated the biological and biochemical effects of cbl-b on EGFR function in MDA-MB-468 breast cancer cells. These cells express high

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levels of EGFR and undergo apoptosis upon EGF treatment (20–22) and thus provide a useful reagent in which to evaluate regulation of EGFR function. Using this well characterized EGF response, we demonstrate that cbl-b inhibits EGFR function by enhancing ubiquitination and degradation of activated receptors.

MATERIALS AND METHODS

Expression constructs. The expression plasmid for cbl-b and the control vector have been previously described (23). The HA-epitope tagged ubiquitin expression plasmid was provided by Dr. Dirk Bohmann (24). The GAS element of the IRF-1 gene fused upstream of the luciferase gene (the pZtkLuc plasmid) used as a STAT reporter construct was provided by Dr. Richard Pine (25).

Immunoblotting and immunoprecipitation. Immunoblotting and immunoprecipitation were performed as previously described (23). Rabbit polyclonal anti-cbl-b (H121; Santa Cruz Biotechnology), anti-EGFR (1005; Santa Cruz Biotechnology), anti-HA (Y-11; Santa Cruz Biotechnology), HRP-conjugated anti-phosphotyrosine (4G10; Upstate Biotechnology), phospho-specific anti-MAP kinase antibody (9101; New England Biolabs), and anti-MAP kinase antibody (SC154, Santa Cruz) were used for immunoblotting. Mouse monoclonal anti-EGFR antibody (Ab-3; Oncogene Science) was used for immunoprecipitation of the EGFR.

Cell culture. MDA-MB-468 breast cancer cells were obtained from the ATCC and maintained in culture in RPMI 1640 supplemented with 10% fetal calf serum (FCS) and 1% Penicillin-Streptomycin. To assess EGF induced apoptosis, cells were plated at 10^4 cells/well in 96 well microtiter plates in RPMI 1640 supplemented with 1% FCS and allowed to adhere to the plate overnight. Human recombinant EGF (Collaborative Biomedical Products) was added, the cells were incubated for three days, and viability was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) dye reduction assay as described previously (26). Each EGF concentration was performed in eight wells and data represent the viability of EGF treated cells compared to the viability of untreated cells as a percentage. In experiments to assess the effects of proteosomal inhibition on apoptosis, lactacystin (Calbiochem) was added to the plates at the same time as the EGF. The data represent the viability of EGF plus lactacystin treated cells compared to the viability of cells in lactacystin alone as a percentage.

To measure the biochemical effects of EGF stimulation, MDA-MB-468 cells were grown to 50–70% confluence, starved overnight in RPMI 1640 supplemented with 1% FCS, EGF (100 ng/ml) was added and then cell lysates were prepared as previously described (23). Ubiquitination was measured by transiently transfecting the stable clones with the HA-epitope tagged ubiquitin expression vector and 48 h post transfection starving and stimulating the cells as above. To assess the biochemical effects in the presence of lactacystin, cells were starved as above, lactacystin (5 μM) was added two hours prior to EGF, and lysates were prepared.

To measure STAT activation, 3×10^5 cells/well were plated in six well plates, allowed to adhere overnight, and then transiently transfected with the pZtkLuc plasmid using Lipofectamine (Life Technologies). After transfection, the cells were incubated with or without EGF (100 ng/ml) for 16 h in RPMI 1640 supplemented with 1% FCS. Cells were lysed in Cell Lysis Reagent (Pharmingen) and the luciferase activity in each sample was determined using Luciferase Assay Reagent (Pharmingen) and a Monolight 2010 luminometer (Analytical Luminescence Laboratory).

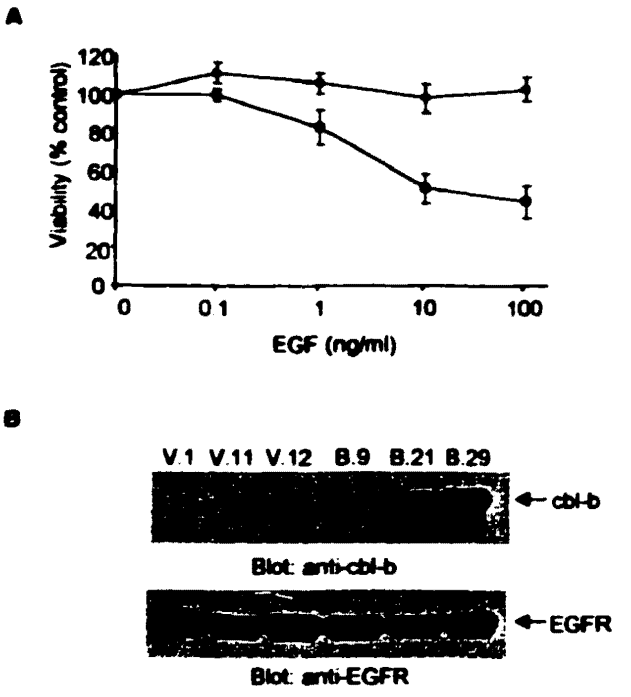

FIG. 1. cbl-b inhibits EGF induced growth inhibition in MDA-MB-468 cells. (A) Stable clones of MDA-MB-468 cells expressing either cbl-b (open circles) or empty vector (closed squares) were cultured with the indicated concentration of EGF for three days and viability was assessed by MTT assay. Data represent the average viability of EGF treated cells as a percentage of untreated cells ± SEM for three clones each of cbl-b and vector controls. (B) Expression of cbl-b and the EGFR in cell lysates from the stable clones was measured by immunoblotting. The positions of the cbl-b and EGFR proteins are indicated by the arrows on the right.

RESULTS

cbl-b inhibits EGF induced apoptosis in MDA-MB-468 cells. To investigate the biological effects of cbl-b on EGFR function, stable clones of MDA-MB-468 breast cancer cells overexpressing cbl-b were isolated.

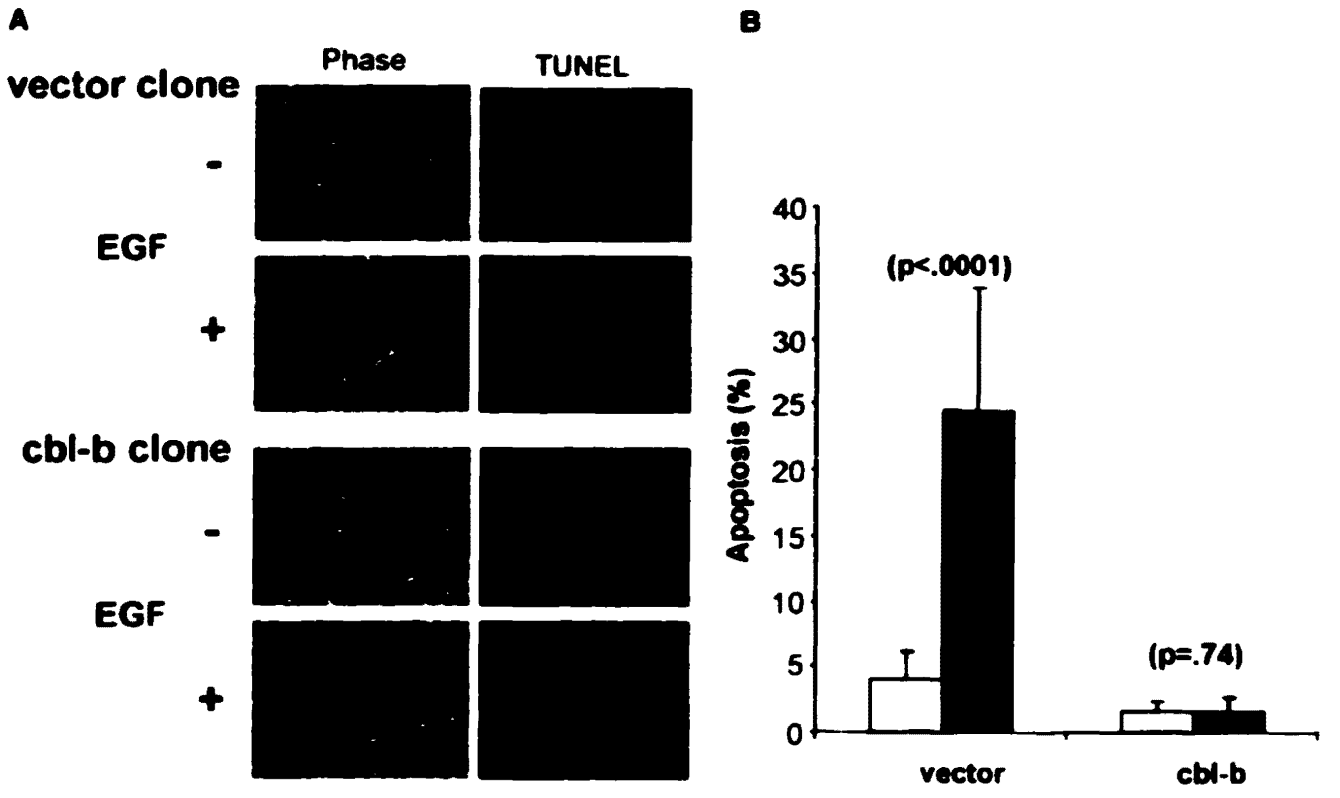

FIG. 2. cbl-b inhibits EGF induced apoptosis in MDA-MB-468 cells. (A) TUNEL assays were performed on cytospins of cbl-b and vector clones which had been incubated ± EGF (100 ng/ml) for 48 h. Phase and fluorescence (TUNEL) photomicrographs (1000×) of the same field for each sample are shown. (B) TUNEL assays were quantitated by calculating the percentage of apoptotic cells in multiple high power fields for a cbl-b and vector clone incubated in the absence (white bars) or presence (black bars) of EGF. A minimum of 500 cells were counted for each condition. Representative data of one experiment are shown. *p* values compare EGF treated and untreated cells for each clone using a Student's two-tailed *t* test.

Overexpression of cbl-b in MDA-MB-468 cells inhibited EGF induced apoptosis (Fig. 1). EGF treatment of stable clones of MDA-MB-468 cells transfected with empty vector results in a dose dependent growth inhibition as measured by MTT with a maximal inhibition of 50–60%. In contrast, cells stably overexpressing cbl-b were not growth inhibited by EGF (Fig. 1A). There was no difference in the growth of MDA-MB-468 clones overexpressing cbl-b compared to vector controls when the cells were cultured in complete growth media in the absence of EGF. Figure 1B demonstrates the expression of cbl-b and the EGFR in the stable clones used for these experiments. A longer exposure of the immunoblot than the one shown revealed that there was endogenous cbl-b in the MDA-MB-468 cells and the cbl-b clones had >20- to 30-fold overexpression. There was no difference in the total level of EGFR in the cbl-b clones compared to the vector controls when the cells were grown in complete growth medium without EGF (Fig. 1B). Analysis of cell surface receptors by cell surface labeling also showed no differences between the cbl-b clones and vector controls (data not shown). Thus, the inhibition of EGFR function by cbl-b was not due to lower steady state expression levels of EGFR by the cbl-b clones.

Previous work has shown that the inhibition of growth by EGF in MDA-MB-468 cells is due to a dose dependent induction of apoptosis by EGF (21). Microscopic analysis revealed that many of the vector control MDA-MB-468 cells treated with EGF became nonadherent, shrunken, refractile cells with pyknotic nuclei compared to untreated cells (data not shown). TUNEL assay to detect apoptotic cells (27) demonstrated that the morphologic changes seen in the EGF treated control cells were due to apoptosis and that EGF treatment was accompanied by an ~6-fold increase in the percentage of cells undergoing apoptosis (Fig. 2). In contrast, cells overexpressing cbl-b did not undergo any morphological changes in the presence of EGF and TUNEL assay demonstrated that there was no change in the percentage of cbl-b cells undergoing apoptosis in the presence of EGF (Fig. 2).

cbl-b inhibits signaling by the EGFR. To investigate the biochemical effects of cbl-b on the activation of the EGFR, cell lysates were prepared from MDA-MB-468 cells overexpressing cbl-b and a vector control over a time course of EGF treatment (Fig. 3). The activation of the EGFR, measured by tyrosine phosphorylation of the receptor, was similar at early time points (e.g.,

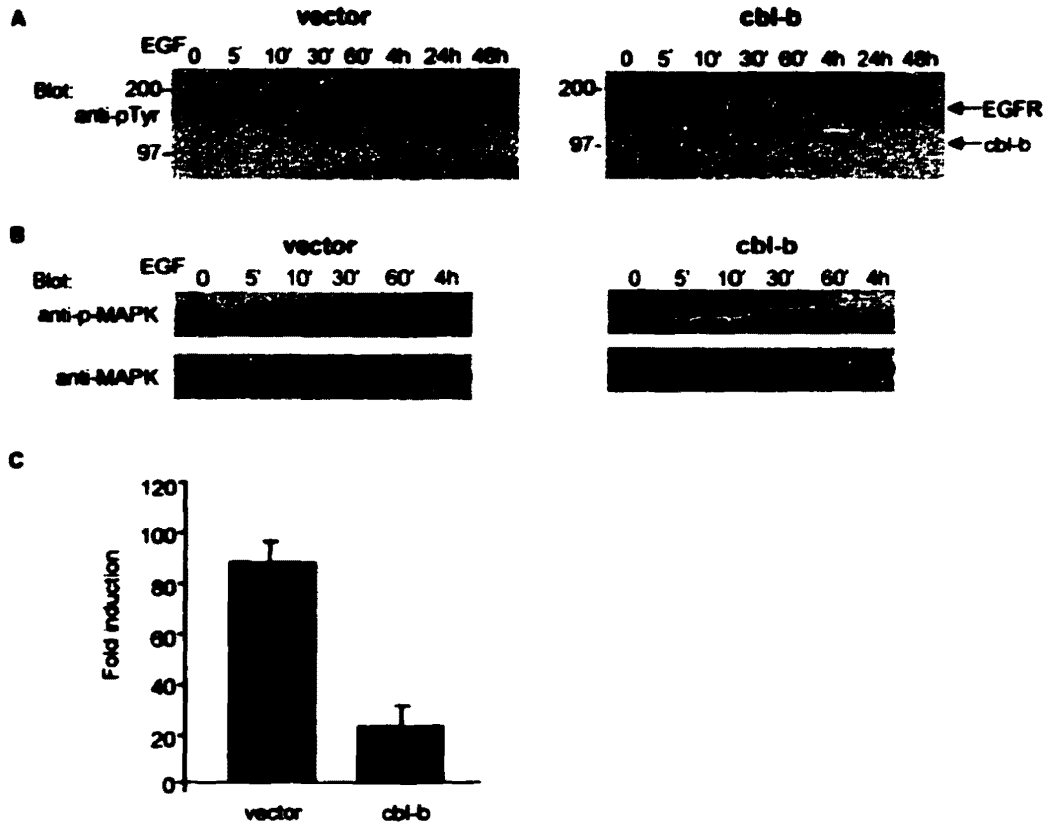

FIG. 3. cbl-b inhibits EGFR signaling. (A) A cbl-b and vector clone were stimulated with EGF (100 ng/ml) for the times indicated and cell lysates were prepared. Proteins were separated by SDS–PAGE, transferred, and immunoblotted with an anti-phosphotyrosine (anti-pTyr) antibody. The positions of the EGFR and cbl-b are indicated by the arrows to the right. The molecular weights in kDa are shown to the left of the figure. Identical results were obtained with other clones. (B) Lysates from the cbl-b and vector clones described above were immunoblotted with an anti-phospho-MAPK antibody (anti-p-MAPK). The filters were stripped and reblotted with an anti-MAPK antibody (anti-MAPK). Identical results were obtained with other clones. (C) Vector and cbl-b clones were transiently transfected with a luciferase reporter plasmid for STAT activation. The cells were incubated ± EGF (100 ng/ml) for 16 h and the luciferase activity was measured. Each experiment was performed in triplicate and the fold induction was calculated as the ratio of luciferase activity in the presence of EGF divided by luciferase activity in the absence of EGF. The results represent the average ± SD for a representative experiment. Identical results were obtained with other clones.

5–30 min) in the cells overexpressing cbl-b and vector control cells (Fig. 3A). However, the activated receptor began to decrease in the cbl-b clone after 1 h and had returned to baseline by 48 h. In contrast, the activation of the EGFR in the vector control cells persisted at a high level throughout the time course.

cbl-b becomes rapidly phosphorylated and recruited to the EGFR in response to EGFR activation (23) and the appearance of a phosphoprotein at the size of cbl-b can be seen in the cbl-b clone as early as 5 min after EGF stimulation (Fig. 3A). The phosphorylation of cbl-b decreases at the later time points, consistent with a loss of EGFR activity. Upon longer exposure, a weaker signal representing a phosphoprotein that migrates at the size of cbl-b (representing endogenous c-cbl or cbl-b protein) can be seen in the vector controls and the phosphorylation persisted at constant levels until 24 h without decrease.

To further investigate the effects cbl-b on downstream signaling by the EGFR, the activation of MAP kinase (MAPK) in cells overexpressing cbl-b and in vector controls was measured (Fig. 3B). EGF treatment induced rapid activation (within 5 min) of MAPK in cells expressing cbl-b and in the vector control cells. However, the magnitude of MAPK activation was less and the duration of activation was shorter in cells expressing cbl-b compared to the vector control cells. Previous work has established that STAT activation is critical to the induction of apoptosis by the EGFR (22, 28). STAT activation was measured by transiently transfecting the cells with a reporter construct consisting of the GAS element of the IRF-1 promoter cloned upstream of the luciferase gene (25). Cells overexpressing cbl-b had significantly lower induction of STAT activity upon EGF treatment (Fig. 3C).

Together, these data demonstrate that overexpression of cbl-b decreases the duration of activation of the EGFR and of multiple downstream signaling pathways.

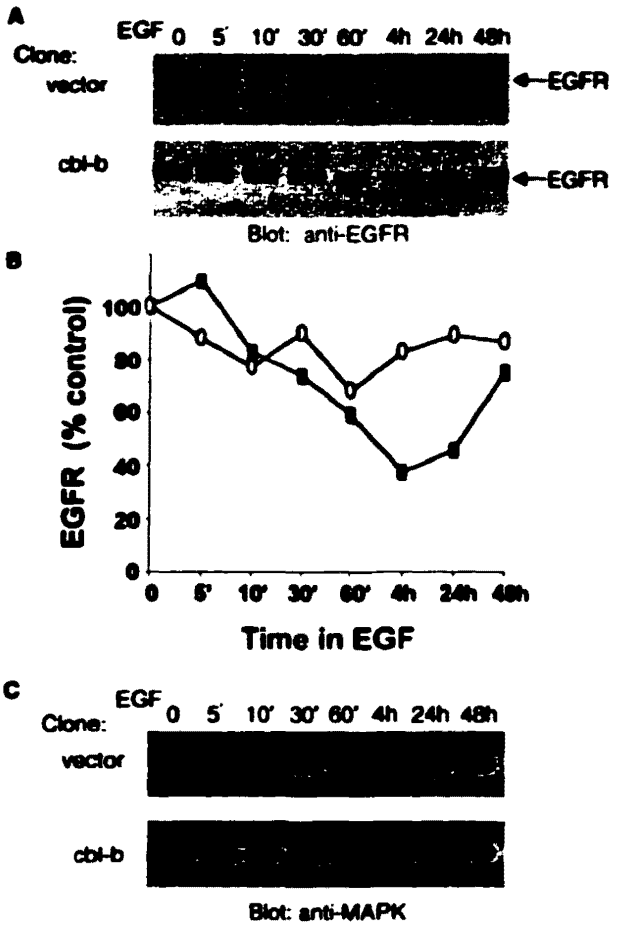

FIG. 4. cbl-b enhances EGF induced degradation of the EGFR. (A) Lysates prepared as described in Fig. 3 were immunoblotted with anti-EGFR antibody. The position of the EGFR is indicated by the arrow to the right. (B) The level of the EGFR receptor, expressed as a percentage of the level in untreated cells (control), was determined by densitometry for the cbl-b clone (black squares) or the vector clone (open circles) from the blot shown in B. (C) Loading was assessed for the blots in B by stripping the blots and reprobing them with an anti-MAPK antibody.

cbl-b enhances EGF induced ubiquitination and degradation of the activated EGFR. The inhibition of multiple signaling pathways by cbl-b and its recruitment to the activated EGFR (23) suggested that the inhibitory function of cbl-b was likely to target the EGFR directly. Previous work has shown that the EGFR is internalized and degraded upon activation and that this activation dependent degradation is enhanced by c-cbl overexpression (18). We therefore investigated the effects of cbl-b overexpression on EGFR degradation. The level of EGFR upon EGF treatment was assessed by immunoblotting (Fig. 4). There was a significant decrease in the level of the EGFR over the first 24 h of EGF treatment in the cbl-b clone and then the levels returned to the baseline (Fig. 4A). Quantitation of the levels by densitometry revealed that the level of the EGFR decreased to approximately 40% of the initial level (Fig. 4B). In contrast, the level of the EGFR did not decrease below 70% of the initial value in the vector control cells. Interestingly, the increase in EGFR levels in the cbl-b clone at 48 h (Fig. 4A) was unphosphorylated EGFR (Fig. 3A) and thus inactive receptor. Analysis of cell surface receptors by cell surface labeling confirmed these results (data not shown). Equal loading of the blots was demonstrated by reprobing the blots with an anti-MAPK antibody (Fig. 4C).

A prominent high molecular weight smear above the EGFR, characteristic of the addition of polyubiquitin chains to the activated EGFR (18), is seen upon EGF treatment in the cells overexpressing cbl-b (Figs. 3A and 4A). To demonstrate that cbl-b enhances ubiquitination of the activated EGFR in these cells, HA-epitope tagged ubiquitin (24) was transiently transfected into either cbl-b overexpressing cells or vector control cells. The cells were incubated with or without EGF and the EGFR was immunoprecipitated from lysates prepared from these cells (Fig. 5). In both the immunoblot of the lysates and the immunoprecipitated EGFR, the anti-HA antibody detected a diffuse ubiquitinated band in the EGFR from EGF-treated cells overexpressing cbl-b (Fig. 5, top panels). This band migrated at the same size as the high molecular weight smear seen above the EGFR when the blot was reprobed with the anti-EGFR antibody (Fig. 5, bottom panels). Longer exposures of the EGFR from vector control cells demonstrate a less prominent smear that reacted with the anti-HA antibody, thus showing that there was less ubiquitination of the EGFR in these cells. Stripping and reprobing the same immunoblots with an anti-ubiquitin antibody showed identical results (data not shown). Together, the data in Figs. 4 and 5 demonstrate that cbl-b enhances ubiquitination and degradation of the EGFR.

Inhibition of the proteosome reverses the effects of cbl-b overexpression. Ubiquitination of proteins targets them for degradation by the 26S proteosome (29) and previous work has established that the EGFR is degraded at least partially by this mechanism (18). To confirm that enhanced ubiquitination and degradation of the activated EGFR was the mechanism by which cbl-b inhibits the effects of the EGFR, we repeated the experiments described above in the presence of lactacystin, a specific inhibitor of proteosomal degradation (30) (Fig. 6). As above, in the absence of lactacystin, EGF did not induce apoptosis in MDA-MB-468 cells overexpressing cbl-b (Fig. 6A, 0 point). However, in the presence of lactacystin, EGF induced apoptosis in the cbl-b overexpressing cells to a level similar to the control cells shown in Fig. 1A. Biochemical analysis revealed that preincubation with lactacystin inhibited the degradation of the EGFR seen in the cbl-b clone (Fig. 6B). In the absence of lactacystin, EGF induced a decrease in the EGFR levels to ~30% of the initial level by 2 h but in the presence of lactacystin the EGFR was

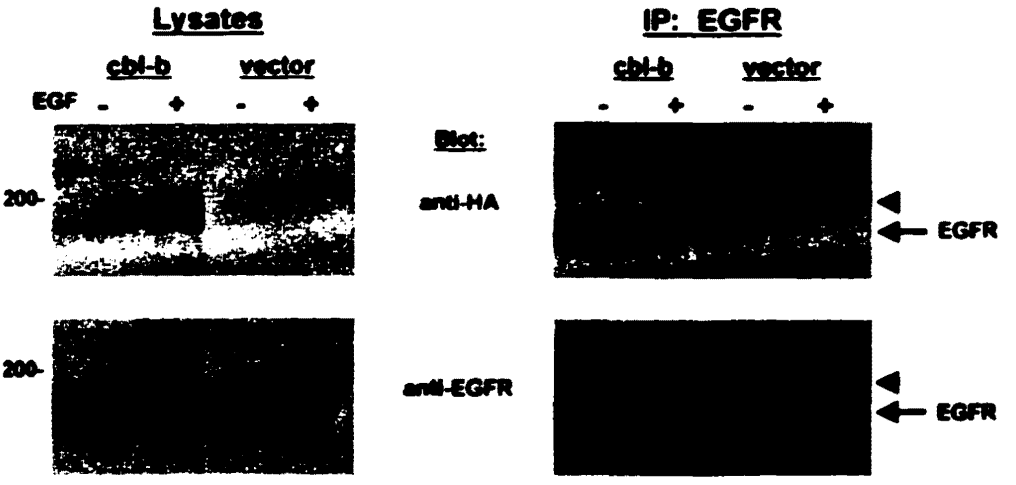

FIG. 5. cbl-b enhances ubiquitination of the EGFR. A cbl-b and vector clone were transiently transfected with HA-epitope tagged ubiquitin. Cells were incubated ± EGF (100 ng/ml) for 10 min and cell lysates were prepared. The EGFR was immunoprecipitated from each lysate. Both the lysate and the precipitated proteins were separated by SDS–PAGE and immunoblotted with an anti-HA antibody (top). The same filter was stripped and reprobed with the anti-EGFR antibody (bottom). The arrows on the right indicate the position of the EGFR and the arrowheads indicate the position of the ubiquitinated EGFR.

maintained at ~60% of the initial levels. Activation of the EGFR in the cbl-b clone, measured by tyrosine phosphorylation of the receptor, was sustained in cells treated with lactacystin compared to the untreated cells (Fig. 6C). Downstream signaling was also maintained in the presence of the proteosomal inhibitor. The phosphorylation of cbl-b (the ~120 kDa band in Fig. 6C) and the phosphorylation of MAPK (Fig. 6D) was sustained longer in the cells treated with lactacystin than in the untreated cells.

DISCUSSION

The data presented here demonstrate that cbl-b inhibits EGF induced apoptosis in MDA-MB-468 cells. cbl-b does not prevent the initial activation of the EGFR and subsequent downstream signaling (Fig. 3) but it does shorten the duration of EGFR signaling. In our previous work, we have shown that cbl-b inhibits EGFR dependent growth and there too the duration of EGFR signaling was shortened (23). Thus, the inhibition of EGFR function is independent of the biological result of EGFR activation. The shortened duration of activation of the EGFR and multiple downstream pathways seen in both systems further suggested that the negative regulatory effects of cbl-b on EGFR function occurred at the level of the EGFR itself.

cbl-b inhibits EGFR function by enhancing ubiquitination and degradation of the activated EGFR (Figs. 4 and 5). There was no change in the steady state level of EGFR in the MDA-MB-468 cells which overexpress cbl-b when the cells were cultured in the absence of EGF. In contrast, upon activation of the EGFR, there was a decrease in the level of EGFR and concomitantly there was almost total loss of activated EGFR. At later time points, the level of EGFR returned to the baseline level but the receptor was unphosphorylated and thus inactive. These observations indicate that overexpression of cbl-b enhances the degradation of the activated EGFR but does not cause degradation of unstimulated receptors. This directly leads to an abrogation of EGFR activation and inhibition of downstream signaling pathways and thus results in inhibition of the biologic effects of EGF.

Levkowitz *et al.* (18) have recently demonstrated that c-cbl enhances ubiquitination and degradation of the activated EGFR. We have recently demonstrated that a third member of the mammalian cbl family, cbl-3, also inhibits EGFR signaling (4) and it too enhances degradation of the activated EGFR (unpublished observation). Thus, regulation of EGFR signaling by degradation is a conserved function of all known mammalian cbl proteins. The biochemical mechanism by which cbl proteins causes ubiquitination of the EGFR is not yet known. Ubiquitination of proteins occurs via the activation and conjugation of ubiquitin to target proteins by a series of enzymes known as E1, E2, and E3 proteins (29). The E3 component is often comprised of a complex of several proteins and confers specificity to the ubiquitination process. Recently, a number of proteins containing a RING finger have been demonstrated to function as E3 proteins or as part of E3 complexes (31–37). The cbl proteins all contain a RING finger and are recruited to the EGFR upon activation (4, 10, 23). The RING finger of c-cbl has been shown to be essential for the ubiquitination and degradation of the EGFR (38). Thus cbl proteins are likely to be part of an E3 complex.

The cbl proteins are substrates in many signaling pathways mediated by tyrosine kinases (9, 10) and

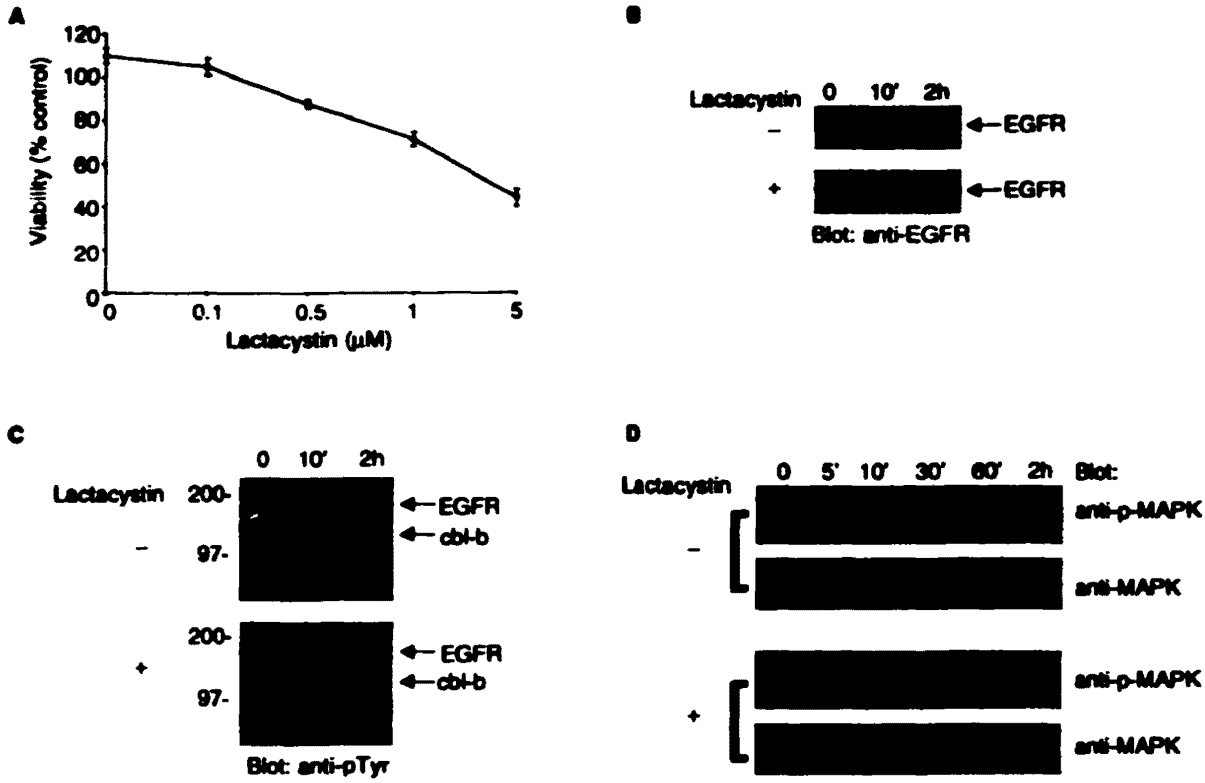

FIG. 6. Lactacystin reverses the EGFR inhibition of cbl-b. (A) A cbl-b clone was incubated with lactacystin at the concentrations indicated in the presence or absence of EGF (100 ng/ml) for 3 days and then viability was assessed by MTT. Viability of cells in lactacystin + EGF as a percent of the viability of the cells in the same concentration of lactacystin without EGF (control) is plotted. Data represent the average ± SD for a representative experiment. (B) A cbl-b clone was preincubated ± lactacystin (5 μM) for 2 h, the cells were stimulated with EGF for the times indicated, and cell lysates were prepared. The protein was separated on SDS–PAGE and immunoblotted with an anti-EGFR antibody. The position of the EGFR is indicated by the arrows on the right. (C) Protein lysates prepared from EGF stimulated cells ± lactacystin as described above were immunoblotted with anti-pTyr. The positions of the EGFR and cbl-b are indicated by the arrows on the right. The molecular weight in kDa is shown on the left of the figure. (D) Protein lysates prepared from EGF stimulated cells ± lactacystin as described above were immunoblotted with anti-p-MAPK. The filters were stripped and reprobed with anti-MAPK.

c-cbl has been demonstrated to enhance ubiquitination and degradation of other growth factor receptors (16, 17, 19). Our data, together with the observations above, imply that cbl proteins regulate the biological responses to stimulation by a variety of growth factors through degradation of activated growth factor receptors.

ACKNOWLEDGMENTS

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Paper 3

cbl-b Dependent Coordinated Degradation of the Epidermal Growth Factor Receptor Signaling Complex

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Running Title: cbl-b regulates degradation of the EGFR complex

[1]The abbreviations used are: EGF, epidermal growth factor; E3, ubiquitin protein ligase; EGFR, epidermal growth factor receptor; FCS, fetal calf serum; GST, glutathione-S-transferase; HA, hemagglutinin; PBS, phosphate buffered saline; SDS-PAGE, SDS polyacrylamide gel electrophoresis; tyrosine kinase binding (TKB); YVAD-CMK, Tyr-Val-

Ala-Asp-chloro-methyl ketone.

SUMMARY

cbl proteins function as ubiquitin protein ligases for the activated Epidermal Growth Factor Receptor and thus negatively regulate its activity. Here we show that cbl-b is ubiquitinated and degraded upon activation of the receptor. Epidermal Growth Factor induced cbl-b degradation requires intact RING finger and tyrosine kinase binding domains and requires binding of the cbl-b protein to the activated Epidermal Growth Factor Receptor. Degradation of both the Epidermal Growth Factor Receptor and the cbl-b protein is blocked by lysosomal and proteasomal inhibitors. Other components of the Epidermal Growth Factor Receptor signaling complex (i.e. Grb2 and Shc) are also degraded in an Epidermal Growth Factor induced cbl-b dependent fashion. Our results suggest that the ubiquitin protein ligase function of cbl-b is regulated by coordinated degradation of the cbl-b protein along with its substrate. Furthermore, the data demonstrate that cbl-b mediates degradation of multiple proteins in the Epidermal Growth Factor Receptor signaling complex.

INTRODUCTION

The cbl proteins are a family of proteins found in metazoans from nematodes to vertebrates. These proteins have several highly conserved domains including a N-terminal tyrosine kinase binding (TKB) domain and a RING finger (1-9). The three mammalian cbl proteins, c-cbl, cbl-b, and cbl-3 (1,2,6-8), are tyrosine phosphorylated upon activation of a wide variety of growth factor receptors and they associate with many signaling proteins via SH2 and SH3 interactions (reviewed in (10,11)). These diverse interactions modulate signaling through many pathways (10,11). Recent work has shown that c-cbl and cbl-b deficient mice have hyperplastic tissues consistent with a negative regulatory role in cellular proliferation for cbl proteins (12-15). Together, these data indicate that the cbl proteins are important regulators of intracellular signaling and consequently of cell function and development.

cbl proteins are negative regulators of Epidermal Growth Factor Receptor (EGFR)[1] signaling. This was first shown by genetic studies in *C. elegans* which demonstrated that sli-1 (the *C. elegans* cbl homologue) is a negative regulator of the Let-23 receptor tyrosine kinase (the EGFR homologue) in vulva development (3,16). The *Drosophila* cbl protein (D-cbl) has been shown to associate with the EGFR and overexpression of D-cbl in the eye of *Drosophila* embryos inhibits EGFR dependent photoreceptor cell development (4,5). Several studies have shown that mammalian cbl proteins become phosphorylated and recruited to the EGFR upon stimulation (11,17) and that they inhibit EGFR function (7,18-20).

The mechanism underlying the negative regulation of activated tyrosine kinases by cbl proteins has recently been described. cbl proteins function as ubiquitin protein ligases which mediate the ubiquitination of activated tyrosine kinases, including the EGFR, and target them for

degradation (20-31). Ubiquitination of proteins occurs via the sequential activation and conjugation of ubiquitin to target proteins by the ubiquitin activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin protein ligase (E3) (32). The E3 confers specificity to the ubiquitination process. An increasing number of RING finger proteins have been demonstrated to function as E3 proteins or as part of E3 complexes and in each of them the RING finger is essential to this activity (33-43). The highly conserved TKB and RING finger domains of cbl proteins are essential and sufficient for their E3 activity and together these domains target the ubiquitination of activated tyrosine kinases such as the EGFR (20-31).

Here, we show that EGF activation induces a coordinated degradation of the EGFR, cbl proteins, and other proteins of the EGFR signaling complex. These results suggest that cbl proteins regulate degradation of multiple proteins in the active EGFR signaling complex.

EXPERIMENTAL PROCEDURES

Expression Constructs---The expression plasmid for HA epitope tagged cbl-b, c-cbl, and the control vector (pCEFL) have been previously described (18). HA epitope tagged cbl-b N1/3 (aa 1-349), and C2/3 (327-938) were created by PCR amplification and cloning into the pCEFL vector. cbl-b N1/2 (aa 1-483) was created by deleting the C-terminal of the full length wild type cbl-b in pCEFL and in the process the HA epitope was lost. The GST fusion protein for cbl-b N1/2 (aa 29-483) was created by in-frame cloning of a PCR generated N-terminal fragment of cbl-b into pGEX 2TK (Pharmacia). The C373A was created in both the full length wild type cbl-b in pCEFL and in the GST-cbl-b N1/2 fusion protein by site directed mutagenesis (Quick Change Kit; Stratagene). All of the mutant cbl-b constructs were confirmed by sequencing. The EGFR Y1045F mutant expression construct in pcDNA3 has been previously described (26). The GFP expression plasmid (pcDNA3.1/zeo) was obtained from Invitrogen.

Immunoblotting and Immunoprecipitation---Immunoblotting and immunoprecipitation were performed as previously described (18). Anti-AKT (New England Biolabs), anti-cbl-b (H454; Santa Cruz Biotechnology), anti-EGFR (1005; Santa Cruz Biotechnology), anti-ERK-2 antibody (SC154; Santa Cruz), anti-Grb-2 (C-23; Santa Cruz Biotechnology), anti-HA (Y-11; Santa Cruz Biotechnology), HRP-conjugated anti-phosphotyrosine (4G10; Upstate Biotechnology), anti-p85 subunit of PI 3-kinase (06-195; Upstate Biotechnology), and anti-Shc (Transduction Laboratories, Inc.) were used for immunoblotting. Ubiquitination was assayed as previously described using a rabbit polyclonal anti-ubiquitin antibody (44). Mouse monoclonal anti-EGFR antibody (Ab-3; Oncogene Science) and anti-phosphotyrosine (4G10;

Upstate Biotechnology) were used for immunoprecipitation.

Cell Culture---MDA-MB-468 breast cancer cells were obtained from the ATCC and maintained in culture in RPMI 1640 supplemented with 10% fetal calf serum (FCS) and 1% Penicillin-Streptomycin. Stable clones overexpressing wild type and mutant forms of cbl-b were generated as previously described (20). To measure the biochemical effects of EGF stimulation, MDA-MB-468 cells were grown to 50-70% confluence, starved overnight in RPMI 1640 supplemented with 0.5% FCS, and then stimulated with EGF (100 ng/ml; Collaborative Biomedical Products) for the times indicated. To harvest proteins, the cells were washed two times in ice-cold PBS containing 0.2 mM sodium orthovanadate and the cells were lysed in ice-cold lysis buffer (10 mM Tris HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Triton X100, 10% Glycerol, 100 mM iodoacetamide, 2 mM sodium orthovanadate, and protease inhibitors (Complete tabs®, Boehringer Mannheim)). The lysates were cleared of debris by centrifugation at 16,000 x g for 15 min at 4°C. In experiments to rule out sequestration of cbl-b upon EGF stimulation, the cells were lysed in SDS buffer (2% SDS, 20 mM Tris HCl pH 7.5, 50 mM NaCl, 5 μM DTT), boiled for 5 min, sonicated, and then run on SDS-polyacrylamide gels (SDS-PAGE) without clarification of the lysates. To assess the biochemical effects in the presence of protease inhibitors (e.g. lactacystin or NH_4Cl), cells were starved as above, the protease inhibitor was added two hours prior to EGF, and lysates were prepared as above.

293T cells were maintained in culture in DMEM supplemented with 10% FCS and 1% Penicillin-Streptomycin and were transfected with various constructs using calcium phosphate (5 Prime→3 Prime, Inc.) according to the protocol included with the reagents. To measure the

effects of EGF stimulation. 293T cells were grown to 70% confluence, starved overnight in DMEM supplemented with 0.5% FCS, and then stimulated with EGF (100 ng/ml) for the times indicated. Proteins were harvested as describe above for MDA-MB-468 cells.

In Vitro Ubiquitination Assay---Autoubiquitination of cbl proteins was performed as previouly described (40). Briefly, GST or GST fusion proteins were incubated in the presence or absence of recombinant wheat E2 (20 ng; UbcH5B) along with recombinant wheat E1 (20 ng), ^{32}P-labeled ubiquitin ($2x10^4$ cpm), in ubiquitination buffer (50 mM Tris-HCl, pH 7.4, 2 mM adenosine 5'-triphosphate, 5 mM $MgCl_2$, and 2 mM dithiothreitol) at 30°C for 90 min. The reaction mixture was separated by 7.5% SDS-PAGE and visualized with a Storm PhosphoImager and Image Quant software (Molecular Dynamics). After exposure, the gels were stained with Coomassie blue to ensure that similar amounts of GST fusion proteins had been used.

RESULTS

cbl-b is Downregulated Upon EGF Stimulation---In prior work, we demonstrated that overexpressing cbl-b in the MDA-MB-468 breast cancer cell line enhances EGF induced ubiquitination and degradation of the EGFR (20). This results in inhibition of EGFR function. While further investigating activation-induced downregulation of the EGFR by cbl-b in these MDA-MB-468 cells, we observed that there was an activation induced loss of cbl-b that parallels the decrease in the EGFR (Fig. 1A, lanes 1-7). In the absence of cbl-b, the EGFR showed only minimal degradation (Fig. 1A, lanes 8-14). cbl-b degradation did not reflect a general decrease of cellular proteins as Akt and ERK-2 protein levels did not change. These results were confirmed in multiple independent cbl-b and vector clones. This change in protein level was not due to changes in mRNA as the level of exogenous cbl-b mRNA, measured by Northern analysis, was stable throughout the time course of the experiment (Fig. 1B). To demonstrate that the observed decrease in cbl-b protein represented a loss of protein and not sequestration in an insoluble compartment, cell lysates were prepared from EGF starved and stimulated cells by resuspending the cells in a 2% SDS lysing buffer, boiling, and then sonicating the lysates. The protein was run on SDS-PAGE gels without clarification of the lysates. In these lysates, cbl-b protein levels still were observed to decrease upon EGF stimulation (Fig. 1C).

Clones of the MDA-MBA-468 cell line which expressed higher levels of cbl-b demonstrated progressively less EGF induced downregulation of cbl-b (Fig. 2A). This suggested that the mechanism of EGF induced downregulation of cbl-b is saturable. When high levels of cbl-b are expressed (as in clone B.29), EGF induces downregulation of only a small

fraction of the total cbl-b. Thus, changes in the cbl-b protein level are not appreciated in the highest expressors. In contrast all of the cbl-b clones downregulated the EGFR to an equal degree as the clone shown in Fig. 1 (data not shown). This indicates that even the lowest expressing clone has sufficient cbl-b to effectively target the EGFR for degradation. These observations further suggest that there might be a stoichiometric relationship between the levels of cbl-b, the EGFR, and the downregulation of both proteins.

To test whether levels of each protein (EGFR and cbl-b) affected the degradation of the other, a constant amount of the EGFR was co-expressed in 293T cells with varying amounts of cbl-b (Fig. 2B). When 3.0 μg of cbl-b plasmid was transfected along with 1.0 μg of the EGFR plasmid, no EGF induced decrease in cbl-b was observed despite downregulation of the EGFR (Fig. 2B, lanes 1 & 2). In contrast, when 0.3 μg of cbl-b plasmid was transfected along with 1.0 μg of the EGFR plasmid, EGF induced a decrease in the cbl-b protein but no decrease in the EGFR (Fig. 2B, lanes 3 & 4). When the expression of both proteins is titrated optimally, EGF induced downregulation of both proteins is observed (Fig. 2C; lane 4). In addition, to determine whether EGF induced a similar downregulation of c-cbl, c-cbl and EGFR were transiently co-expressed in 293T cells. Activation induced downregulation of c-cbl and the EGFR was observed (Fig. 2C; lane 8). Varying the amounts of c-cbl and the EGFR plasmids transfected yielded results similar to the titration of cbl-b presented in Fig. 2B (data not shown). When the EGFR or the cbl proteins were expressed alone in 293T cells, EGF did not induce downregulation of the respective transfected proteins (Fig. 2C; lanes 2, 6, 10).

To test if EGF induced degradation of endogenous cbl proteins, MDA-MB-468 cells were stimulated with EGF, lysates were prepared, and protein levels assessed by immunoblotting (Fig.

2D). EGF induced degradation of the endogenous c-cbl and cbl-b (Fig. 2D, lane 2). In these cells there was only slight degradation of the EGFR. This result is concordant with the titration data described above (Fig. 2A-2C) since these cells have high levels of the EGFR and relatively low levels of endogenous cbl proteins. These results confirmed that EGF induces degradation of endogenous cbl proteins and further confirmed the stoichiometric relationship described above.

cbl-b is ubiquitinated and Degraded in a Proteasome Dependent Fashion---Degradation of activated EGFRs is sensitive to both proteasome and lysosome inhibitors (22,45,46). Thus, EGFR degradation is dependent on both the proteasomal and lysosomal pathways. To test if cbl-b degradation occurred by a mechanism similar to that of EGFR degradation, MDA-MB-468 cells overexpressing cbl-b were stimulated with EGF in the presence or absence of lactacystin (an inhibitor of proteasomes (47)). Lactacystin inhibited EGF-induced degradation of both cbl-b and the EGFR proteins but had little or no effect on the level of the proteins in the absence of EGF simulation (Fig. 3; top panels). Similar inhibition of EGF-induced degradation was seen when cells were incubated with inhibitors of lysosome function (e.g., ammonium chloride (46)) (data not shown). In contrast, the caspase inhibitor YVAD-CMK, which has been shown to inhibit the degradation of cbl proteins that occurs during apoptosis (48), did not block the EGF induced degradation of cbl-b (Fig. 3; bottom panels). These inhibitor experiments suggested a common degradation pathway for both cbl-b and the EGFR.

The EGFR is targeted for degradation by activation induced ubiquitination mediated by the cbl proteins (20,22,23,25-27). To test if cbl-b is also ubiquitinated, cbl-b was immunoprecipitated from cell lysates starved or stimulated with EGF in the presence of

lactacystin and then immunoblotted with a polyclonal anti-ubiquitin antibody (Fig. 4). In cells overexpressing cbl-b. EGF activation results in the appearance of a smear of anti-ubiquitin immunoreactive species in the cbl-b immunoprecipitates indicative of the addition of multiple ubiquitin molecules to cbl-b (Fig. 4: lane 4). Similarly. EGF induced ubiquitination of the EGFR (Fig. 4. lane 8). Notably. the ubiquitinated forms of cbl-b begin at a smaller size (~125 kDa) than the un-ubiquitinated EGFR (~180 kDa). indicating that the higher molecular weight ubiquitinated proteins are not co-precipitating ubiquitinated EGFRs. Similarly. the ubiquitinated species detected in the EGFR immunoprecipitates migrate above the position of the un-ubiquitinated EGFR (Fig. 4. lane 8). Thus. cbl-b becomes ubiquitinated upon EGF activation.

Structural Requirements for cbl-b Degradation---The ability of cbl proteins to induce ubiquitination and degradation of activated EGFRs requires an intact TKB and RING finger domain (23.25-27). To assess the structural requirements for cbl-b ubiquitination and degradation. stable clones of the MDA-MB-468 cell line expressing mutant forms of cbl-b were generated. EGF activation of cells expressing full length cbl-b or the N-terminal half of cbl-b (which contains both the TKB and RING finger domains) resulted in downregulation of both the cbl-b and EGFR proteins (Fig. 5A). Both of these forms of cbl-b are recruited to the EGFR upon activation. These results also demonstrate that proline rich SH3 docking sites and the UBA domain in the C-terminal half of cbl-b are not required for cbl-b or EGFR downregulation. In contrast. EGF activation of cells expressing mutants of cbl-b which have deletions of either the RING finger or the TKB domain (N1/3 and C2/3 respectively) does not

result in downregulation of either the cbl or EGFR proteins. The N1/3 protein (containing the TKB domain) coimmunoprecipitated with the activated EGFR while the C2/3 protein (lacking the TKB domain) did not. Thus the TKB and RING finger domains are required for downregulation of both cbl-b and the EGFR.

To directly assess the role of the RING finger in activation induced cbl-b downregulation, a point mutation was introduced in the first cysteine of the cbl-b RING finger (C373A). This point mutation resulted in the complete abrogation of EGF induced downregulation of both the EGFR and the mutant cbl-b and an accompanying loss of ubiquitinated forms of both proteins (Fig. 5A and data not shown). To confirm that this mutation destroyed the ability of cbl-b to function as an E3, bacterially produced GST fusion proteins for cbl-b (N1/2) with or without the C373A mutation were tested in an *in vitro* ubiquitination reaction in which the fusion protein serves as the primary substrate (40). As is evident, cbl-b mediates its own ubiquitination while the C373A mutant does not (Fig. 5B). These data establish that the EGF induced ubiquitination and downregulation of cbl-b has the same structural requirements as those needed for EGFR ubiquitination and downregulation. Downregulation of both proteins requires intact cbl-b TKB and RING finger domains.

cbl-b Downregulation Requires Binding to the EGFR---Previous work has shown that EGFR downregulation by cbl proteins requires binding of the cbl protein to the activated receptor (22,23,26,27). The apparent stoichiometric relationship between cbl-b degradation and EGFR degradation (described in Fig. 2) and the structural requirements for both an intact TKB domain and RING finger (described in Fig. 5) suggested that EGF induced downregulation of the cbl-b

protein requires binding of cbl-b to the EGFR. To directly test this, we transiently co-expressed cbl-b with either wild-type EGFR or a mutant EGFR (Y1045F-EGFR) to which cbl proteins cannot bind (Fig. 6A). Previous work has shown that the Y1045F-EGFR has a catalytically active kinase but that cbl proteins do not induce its ubiquitination or downregulation (26). Downregulation of cbl-b and EGFR was observed only when cbl-b was co-expressed with the wild-type EGFR. No downregulation of either protein was observed when cbl-b was co-expressed with the Y1045F-EGFR. cbl proteins become phosphorylated upon activation of the EGFR and this is believed to be mediated by Src family kinases which are activated upon stimulation of the receptor (17). Immunoprecipitation of cbl-b demonstrated that cbl-b became phosphorylated in response to activation of either form of the receptor (demonstrating kinase activity for both forms) but that only the wild-type form of the EGFR co-immunoprecipitated with cbl-b (Fig. 6B). These experiments confirmed that EGF induced downregulation of both cbl-b and the EGFR occurred only when the two proteins can bind to one another.

Downregulation of the EGFR Signaling Complex by cbl-b---Since cbl-b and the EGFR were degraded upon activation of the EGFR, EGF induced downregulation of other components of the active signaling complex also were investigated in the MDA-MB-468 clones (Fig. 7). Grb-2 and Shc were also down regulated upon EGF stimulation in clones that expressed either wild type or N1/2 cbl-b (Fig. 7). In addition, their downregulation was blocked by lysosome inhibitors (e.g. NH_4Cl; Fig. 7) or proteasome inhibitors (data not shown). In contrast, clones expressing the C373A RING finger mutant of cbl-b did not downregulate any components of the signaling complex. Thus, the downregulation of Grb-2 and Shc by cbl-b were dependent upon

the overexpression of cbl-b containing both an intact TKB domain and RING finger. When Grb-2 and Shc were immunoprecipitated from EGF stimulated lysates in the presence of lactacystin, no evidence of ubiquitination or larger forms of the proteins could be demonstrated (data not shown). Interestingly, degradation of the p85 kDa subunit of PI3 kinase was not observed upon EGF stimulation. We and others have shown that the p85 kDa subunit is recruited to the activated EGFR upon stimulation through an interaction with the cbl proteins (18,49,50). Whether this lack of degradation indicates that not all proteins that become recruited to the active signaling complex are degraded or simply that the amount of p85 bound to the EGFR is only a small fraction of the total protein is unknown. ERK-2 was not down regulated upon EGF stimulation demonstrating that these results were not due to a general degradation of signaling molecules.

DISCUSSION

cbl proteins have been shown to function as E3s for activated tyrosine kinases (20-31). Here we show that the cbl-b protein is ubiquitinated and degraded upon EGF stimulation along with the EGFR (Fig. 1, 2 and 4). We also found that c-cbl is similarly degraded upon EGF stimulation (Fig. 2C). c-cbl protein ubiquitination has been described upon activation of the CSF-1 receptor (28,29,51). However, c-cbl is not degraded along with the CSF-1 receptor. EGF induced c-cbl degradation has been noted recently but no characterization of the structural requirements or its significance were reported (52). Our results further demonstrate that EGF induced degradation of cbl-b requires an intact cbl-b TKB domain, an intact cbl-b RING finger, and binding of cbl-b to the EGFR (Fig. 5 and 6). Previous work has demonstrated these same

requirements for cbl mediated degradation of the EGFR (23,25-27). The binding of the cbl protein to the EGFR is required for activation of the E3 activity (26). Whether the subsequent degradation of both the cbl protein and the EGFR occurs while they are bound to one another or if they are degraded separately is unknown (see the discussion below). Other E3 proteins have been shown to ubiquitinate themselves and thus to target themselves for degradation. However there is no evidence as yet that they are degraded along with their target proteins (37,40-42). Our data, showing degradation of an E3 in a coordinated fashion along with its target protein, suggests an additional mechanism for the regulation of E3 activity.

The expression of both EGFR and a cbl protein are required for downregulation of either (Fig. 1 and 2). However, the relative amounts of the cbl protein and the EGFR affect the ability to observe the downregulation of the two proteins (Fig. 2). When cbl proteins are in excess, EGF induces EGFR downregulation with no apparent cbl downregulation. Conversely, when EGFR is in excess, EGF induces cbl protein downregulation with no apparent EGFR degradation. Finally, when the concentrations of both proteins are optimally titrated, activation-induced downregulation of both proteins can be observed (e.g., Fig. 2C). Previous experiments were titrated to maximal EGFR degradation and thus used excess cbl protein (18,22,23,25,27). The dosage effect described above could explain why we and others have not observed a decrease in cbl proteins upon EGF stimulation. Together, these results suggest that the EGFR level determines the amount of degradation of cbl-b (or c-cbl) and that, in the presence of excess cbl-b (or c-cbl), the levels of EGFR are limiting (and vice versa). Thus, the coordinated degradation of cbl-b and the EGFR appears to require a stoichiometric relationship between the two proteins.

We also show that EGF induces degradation of other components of the active signaling

complex (e.g. Grb-2 and Shc) by a cbl-b dependent mechanism (Fig. 7). The cbl-b protein plays a key role in the EGF induced degradation of these other proteins since no degradation is seen in the absence of cbl-b overexpression or when the inactive C373A RING finger mutant of cbl-b is overexpressed. This suggests that the cbl-b protein is regulating the degradation of a signaling complex (including itself) and not simply the degradation of a single target protein. To our knowledge, this is the first description of E3 mediated degradation of multiple components of a receptor signaling complex. Grb-2 can interact directly with the C-terminal half of cbl proteins and the cytoplasmic tail of the EGFR (10,11,53). Shc can interact with both Grb-2 and the EGFR (53). This raises the possibility that these proteins are degraded because of their association with either the EGFR or cbl-b. However, both Grb-2 and Shc are degraded upon EGF stimulation in the cells overexpressing the cbl-b N1/2 protein, which lacks the C-terminal half, suggesting that a direct interaction with the cbl protein may not be necessary.

The mechanism of degradation of the EGFR signaling complex remains to be elucidated. The relationship between ubiquitination of plasma membrane proteins and their internalization, trafficking, and degradation is complex. In yeast, there are membrane proteins which are targeted for degradation in the vacuole by ubiquitination but whose degradation is independent of proteasome function (reviewed in (54)). While many of these proteins are targeted for degradation by poly-ubiquitination, there are clear examples of proteins that are targeted for degradation by mono-, di-, and tri-ubiquitination (54). In mammalian cells, ligand induced internalization and degradation of the growth hormone receptor requires an intact ubiquitinating system, intact proteasome function, and intact lysosome function (55-57). However, ubiquitination of the growth hormone receptor itself is not required for degradation, but

inhibition of proteasome function prevents internalization and degradation of the receptor (57). Similar to the growth hormone receptor, the degradation of the EGFR is blocked by both proteasome and lysosome inhibitors (22,45,46). Previous work suggests that the enhanced ubiquitination of the EGFR by cbl proteins increases sorting of the internalized EGFR to the late endosome where it is degraded by both proteasomal and lysosomal proteases (26). Similarly, we have found that the EGF induced, cbl-b dependent degradation of multiple components of the signaling complex is inhibited by both types of inhibitors (Fig. 3, 7, and data not shown). Again, this supports a common degradation pathway for the entire complex. It has been postulated that the cytoplasmic domains of the EGFR are degraded by the proteasomal proteases and the extracellular domains are degraded by the lysosomal proteases (26). While it is clear that the degradation is dependent on both the proteasome and the lysosome, the mechanism by which the two are coupled remains unknown. It is possible that the entire EGFR signaling complex traffics to the late endosome where it is coordinately degraded by both lysosomal and proteasomal mechanisms. Alternatively, the components of the complex may be separately targeted to either the proteasome and/or lysosome. While our experiments show that the cbl-b protein coordinates the degradation of multiple components of the active EGFR signaling complex, we can not exclude the possibility that other proteins or E3s are also required for this degradation to occur.

EGF induces ubiquitination of both the EGFR and cbl-b (Fig. 4). However, we have been unable to demonstrate EGF induced ubiquitination of the Grb-2 or Shc proteins. It is possible that they do become ubiquitinated but that this is difficult to detect due to low steady state levels of the ubiquitinated species of these proteins. Alternatively, these non-ubiquitinated proteins may be targeted to lysosomal or proteasomal degradation as part of the EGFR signaling complex

because they are associated with other ubiquitinated proteins.

In conclusion, the results above demonstrate that the cbl-b protein is degraded along with its substrate (the EGFR) in a ligand dependent fashion and that cbl-b regulates degradation of multiple proteins in the EGFR signaling complex. Whether the observations we have made are specific to the interaction between cbl proteins and the EGFR or are a general mechanism of cbl protein action remains to be elucidated.

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FIGURE LEGENDS

FIG. 1. **EGF induces downregulation of cbl-b.** *A*. MDA-MB-468 cells expressing cbl-b or a vector control were stimulated with EGF (100 ng/ml) for the times indicated and cell lysates were prepared. Lysates were immunoblotted for cbl-b (HA), EGFR, Akt, or ERK-2 as indicated. Molecular weight standards (in kDa) are shown to the left of the panels. Identical results were obtained with other clones. *B*. Total RNA was prepared from the cbl-b clone stimulated as above and probed for expression of exogenous cbl-b mRNA (top panel). Bottom panel shows ethidium bromide stain of the 28S ribosomal band for loading. *C*. cbl-b cells were starved (-) or stimulated (+) for 8 h with EGF, lysates were prepared as described in the text, and the lysates were immunoblotted for cbl-b (HA) and ERK-2 as indicated.

FIG. 2. **EGF induced degradation of cbl-b and EGFR requires the presence of both proteins.** *A*. Clones of the MDA-MB-468 cell line expressing different amounts of cbl-b were starved (-) or stimulated (+) with EGF (100 ng/ml) for 6 h and lysates were immunoblotted for cbl-b. Loading levels were confirmed to be equal by reprobing the blots with ERK-2 (not shown). *B*. 293T cells were transfected with a fixed amount of EGFR plasmid and varying amounts of cbl-b plasmid as indicated above the panels. Cells were starved or stimulated with EGF for 6 h and lysates were immunoblotted for cbl-b (HA), EGFR, and Green Fluorescent Protein (GFP) as indicated to the right of the panels. *C*. 293T cells were transfected with EGFR plasmid (0.5 μg) and either cbl-b, c-cbl, or empty vector plasmids (0.3 μg) as indicated above the panels. Cells were starved (-) or stimulated (+) with EGF for 6 h and lysates were immunoblotted for cbl-b or c-cbl (HA), EGFR, and GFP as indicated to the right of the panels. GFP was included in the

293T transfections as a control for protein loading and transfection efficiency. *D.* A vector clone of the MDA-MB-468 cell line was starved (-) or stimulated (+) with EGF (100 ng/ml) for 6 h and lysates were immunoblotted for endogenous cbl-b, c-cbl, EGFR, or ERK-2.

FIG. 3. **EGF induced cbl-b and EGFR downregulation is inhibited by lactacystin.** MDA-MB-468 cells expressing cbl-b were pre-incubated for 2 h with (+) or without (-) lactacystin (top panels) or YVAD-CMK (bottom panels) prior to EGF stimulation (100 ng/ml) for 6 h. Lysates were immunoblotted for cbl-b (HA), EGFR, and ERK-2 (for loading) as indicated. Molecular weight standards (in kDa) are shown to the left of the panels.

FIG. 4. **cbl-b is ubiquitinated upon EGF stimulation.** Lysates prepared from a MDA-MB-468 vector or cbl-b clone EGF starved (-) or stimulated (+) in the presence of lactacystin (as described above) were immunoprecipitated for either cbl-b or EGFR as indicated above the panels. The immunoprecipitated protein was immunoblotted with an anti-polyubiquitin antibody (Ub; top panels) and then the filters were stripped and reprobed for cbl-b (HA) or the EGFR (bottom panels). The arrows indicate the position of the un-ubiquitinated cbl-b and EGFR proteins respectively, and the brackets indicate the position of the ubiquitinated forms of the proteins. Molecular weight standards (in kDa) are shown to the left of the panels.

FIG. 5. **Structural requirements for EGF induced cbl-b and EGFR downregulation.** *A.* Clones of MDA-MB-468 expressing wild type cbl-b (Wt), the first one third of cbl-b (N1/3), the first half of cbl-b (N1/2), full length cbl-b with a point mutation in the RING Finger (C373A), or the

last two thirds of cbl-b (C2/3) were starved (-) or stimulated (+) with EGF as described in the legend to Fig. 1 for 6 h. Lysates were immunoblotted for expression of cbl-b or the EGFR as indicated along the top of the figure. The first column shows a schematic representation of the cbl-b proteins expressed in each line. TKB: Tyrosine kinase binding domain; RF: RING finger; PR: proline rich domain; UBA: ubiquitin associated domain. The last column (Bind EGFR) shows the EGF induced binding of each cbl-b protein to the EGFR as determined by co-immunoprecipitation. Equal loading of the immunoblots was confirmed by reprobing the membranes for ERK-2 levels (data not shown). *B*, Bacterially produced GST fusion proteins for the N-terminal half of cbl-b (N1/2), the RING finger mutant of the N-terminal half of cbl-b (C373A), or GST alone were incubated in the presence (+) or absence (-) of recombinant wheat E2 (UbcH5B), recombinant wheat E1, ^{32}P-labeled ubiquitin, and ATP at 30°C for 90 min. The reaction mixture was separated by 7.5% SDS-PAGE and visualized with a Storm PhosphoImager using Image Quant software (Molecular Dynamics). The position of the GST fusion proteins for the N-terminal region of the cbl proteins is indicated by the arrow.

FIG. 6. **cbl-b downregulation requires binding to the EGFR.** *A*, 293T cells were transiently transfected with cbl-b and either wild type EGFR (EGFR) or mutant EGFR (Y1045F). Cells were starved (-) or stimulated (+) with EGF as described in Fig. 2 and lysates were immunoblotted for cbl-b (HA), EGFR, or GFP as indicated to the right of the figure. GFP was included in the 293T transfections as a control for protein loading and transfection efficiency. *B*, cbl-b was immunoprecipitated from the lysates with anti-HA antibody and the immunoprecipitates were immunoblotted for phosphotyrosine (p-Tyr) or cbl-b (HA). The

positions of the EGFR and cbl-b are indicated by arrows to the left of the figure.

FIG. 7. **cbl-b regulates degradation of the EGFR signaling complex.** MDA-MB-468 cells expressing empty vector, wild type cbl-b, the N-terminal half of cbl-b (N1/2), or the RING finger mutant of cbl-b (C373A) were EGF starved (-) or stimulated (+). Lysates were immunoblotted for Grb2, Shc, the 85 kDa subunit of PI3K and ERK-2 as indicated to the right of the panels. Results for incubation with the lysosomal inhibitor ammonium chloride (NH_4Cl) at 10 mM are shown for wild-type and N1/2 cbl-b. Inhibitor experiments are not shown for vector controls or for the C373A mutant since no degradation was seen in the absence of inhibitor.

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Fig. 1

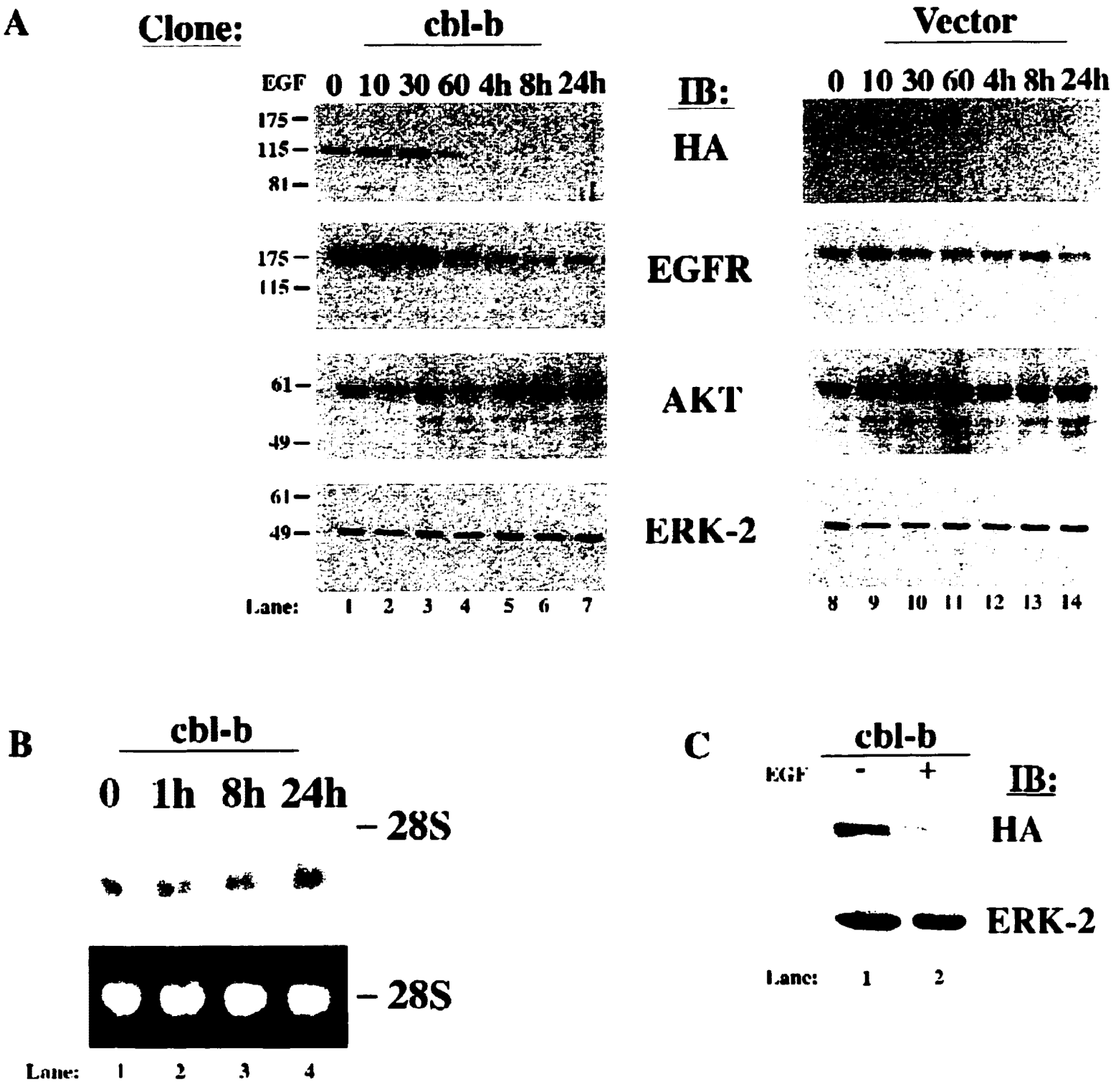

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Fig. 2

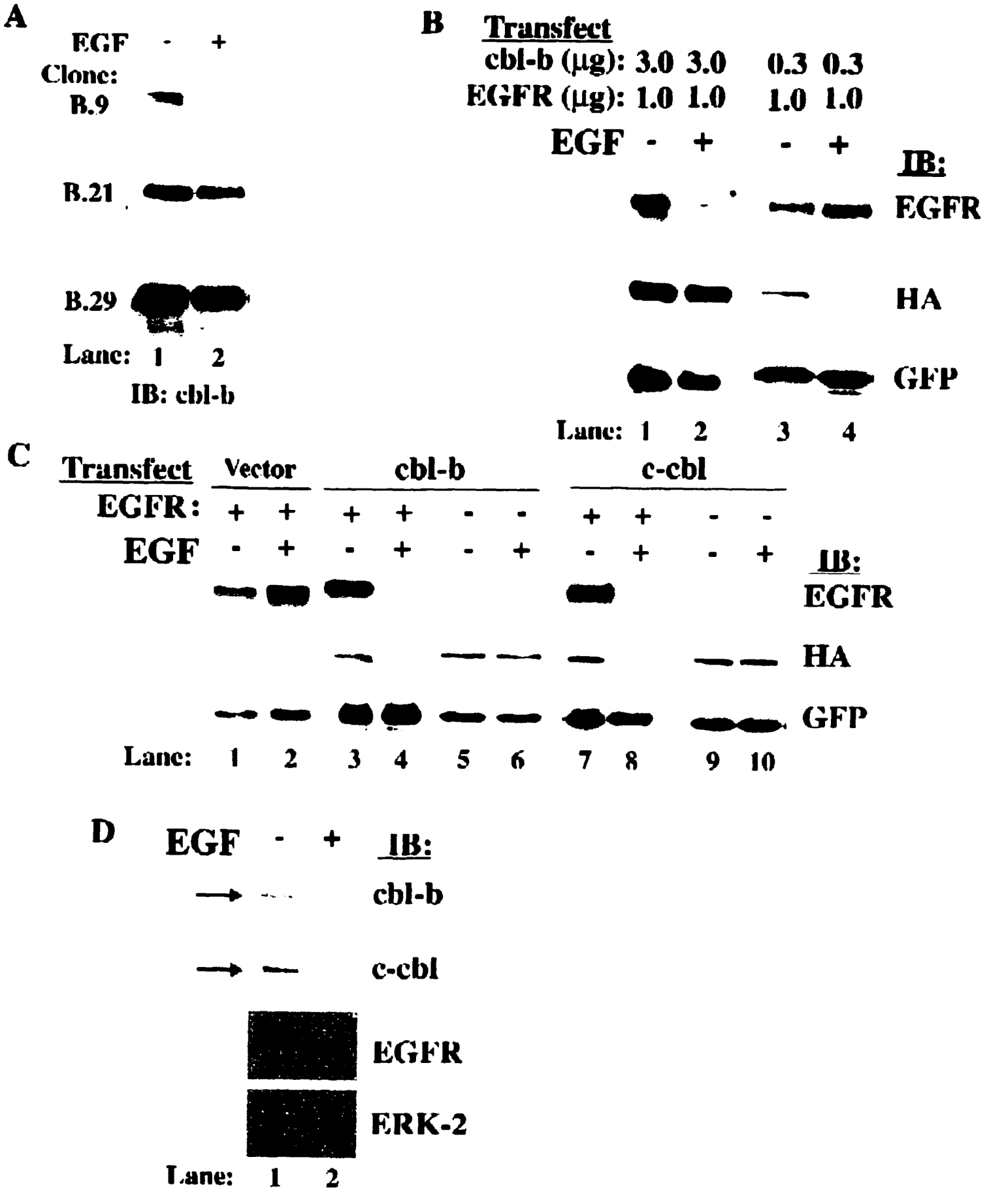

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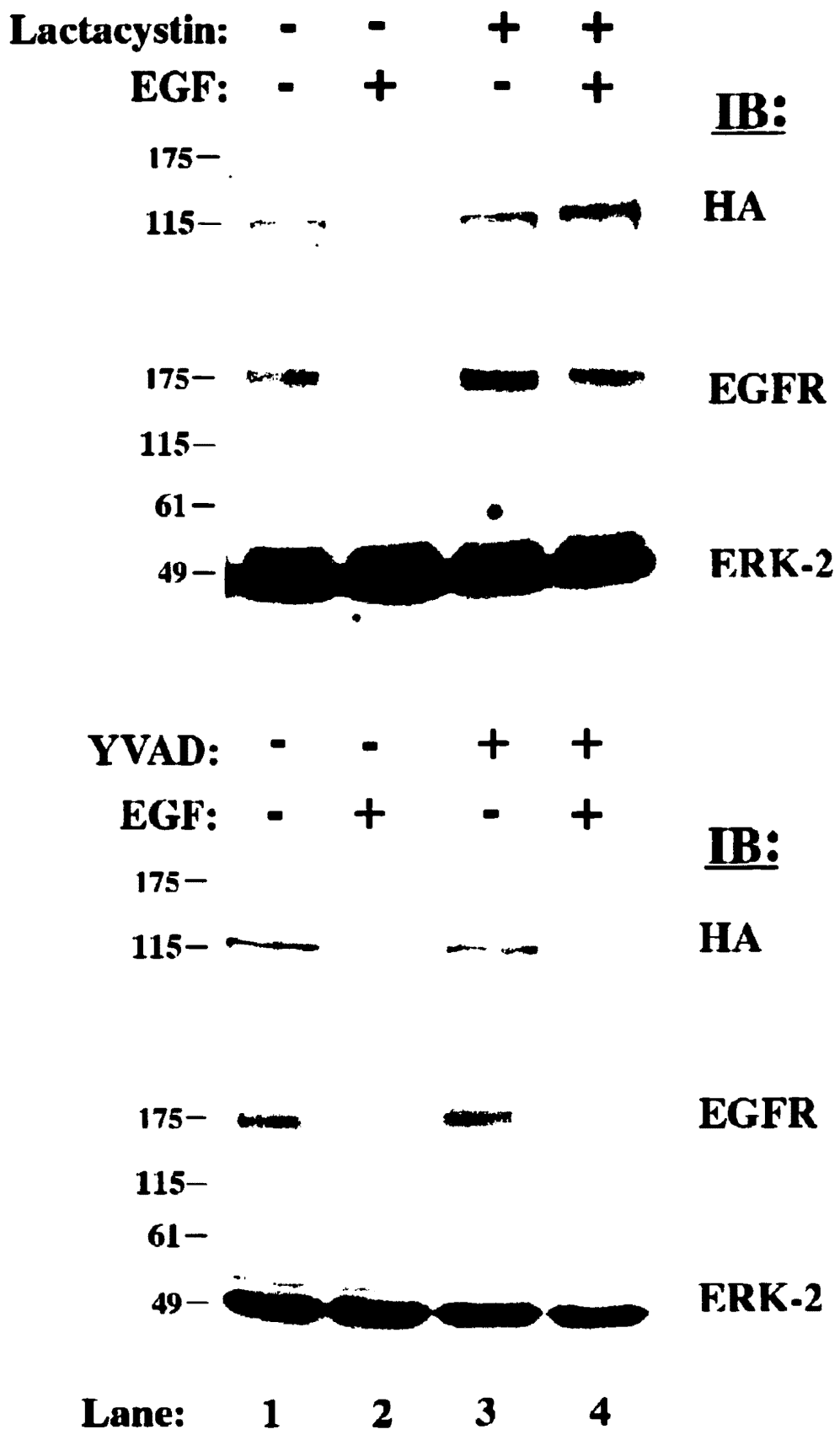
Fig. 3



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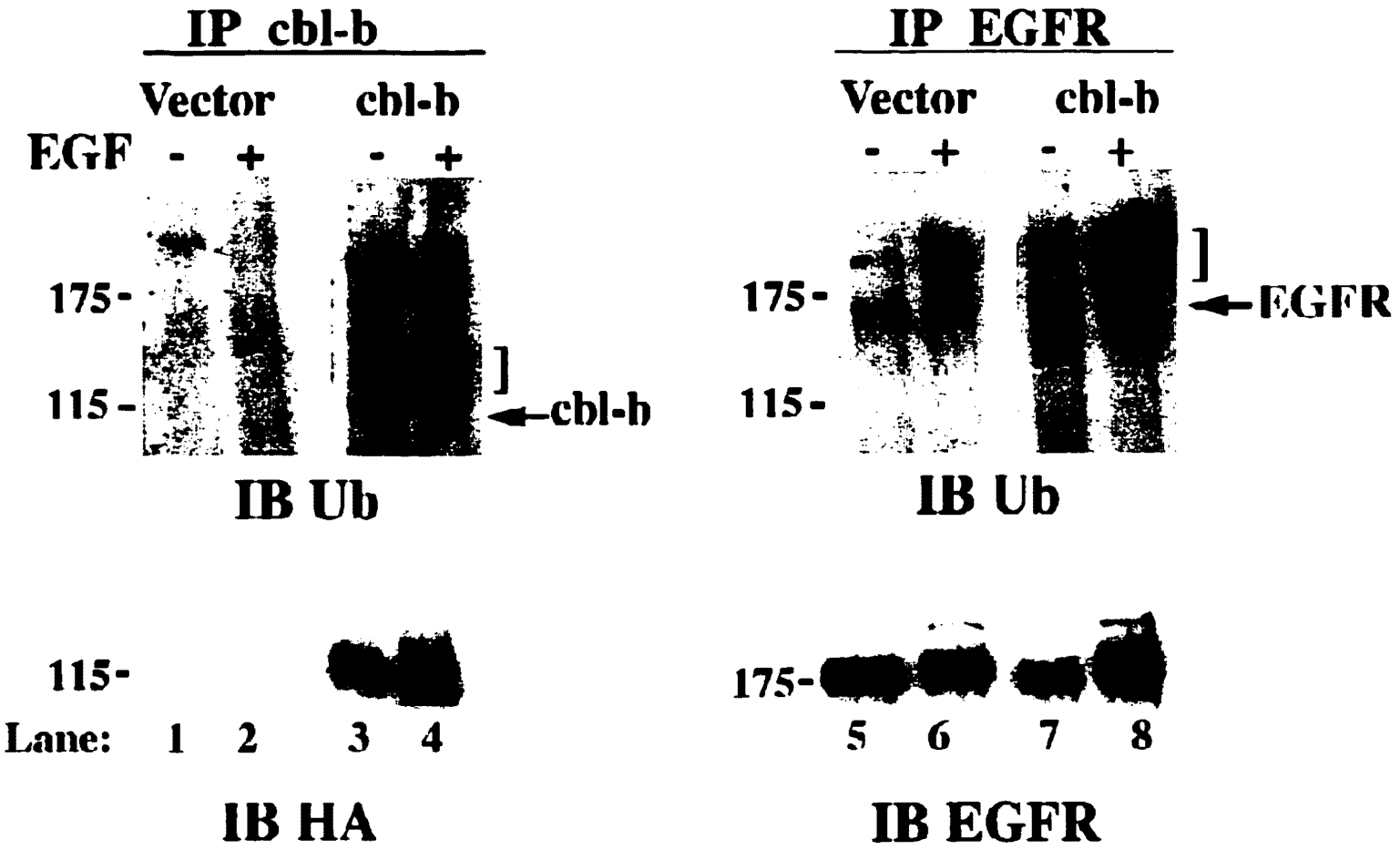

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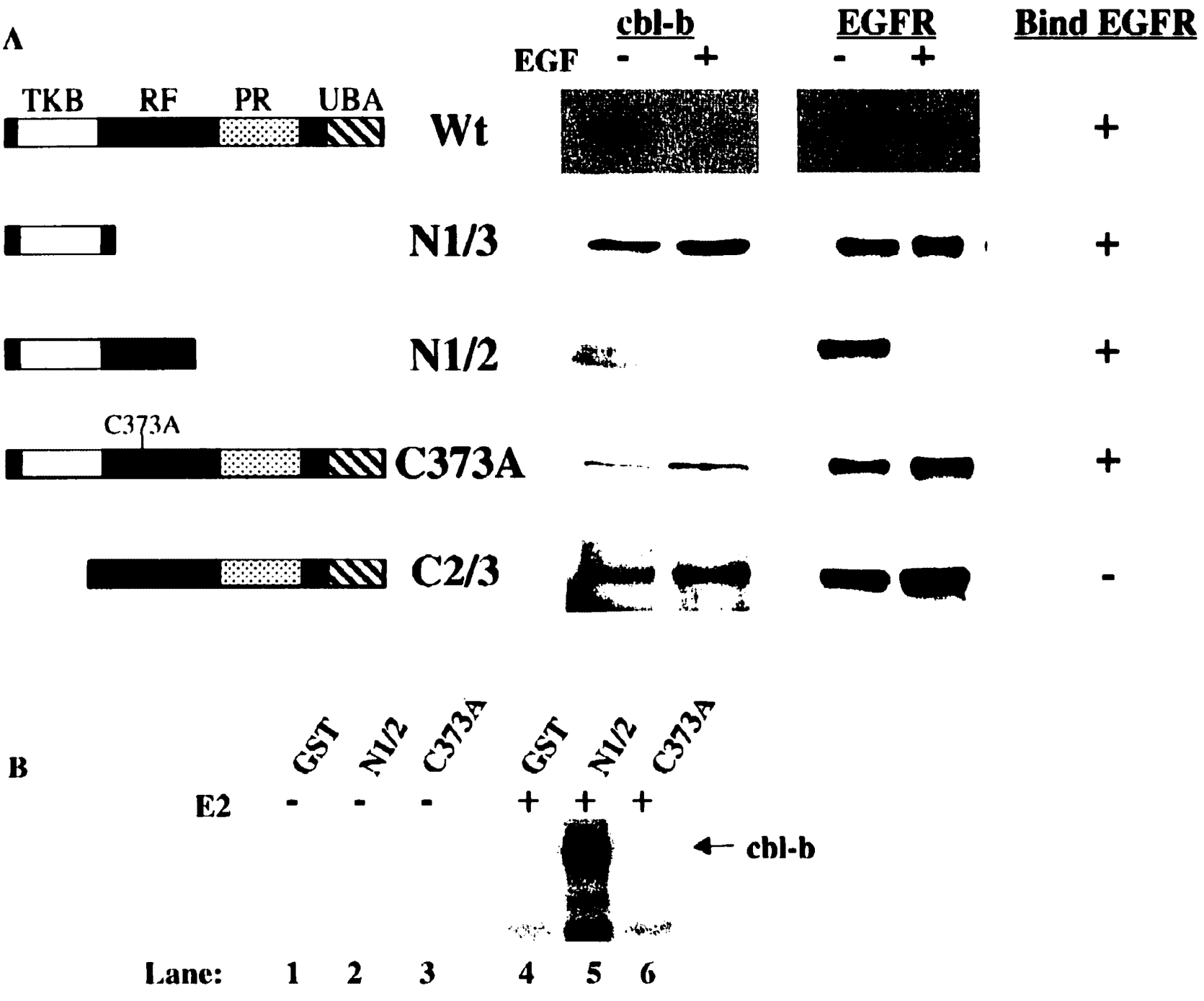

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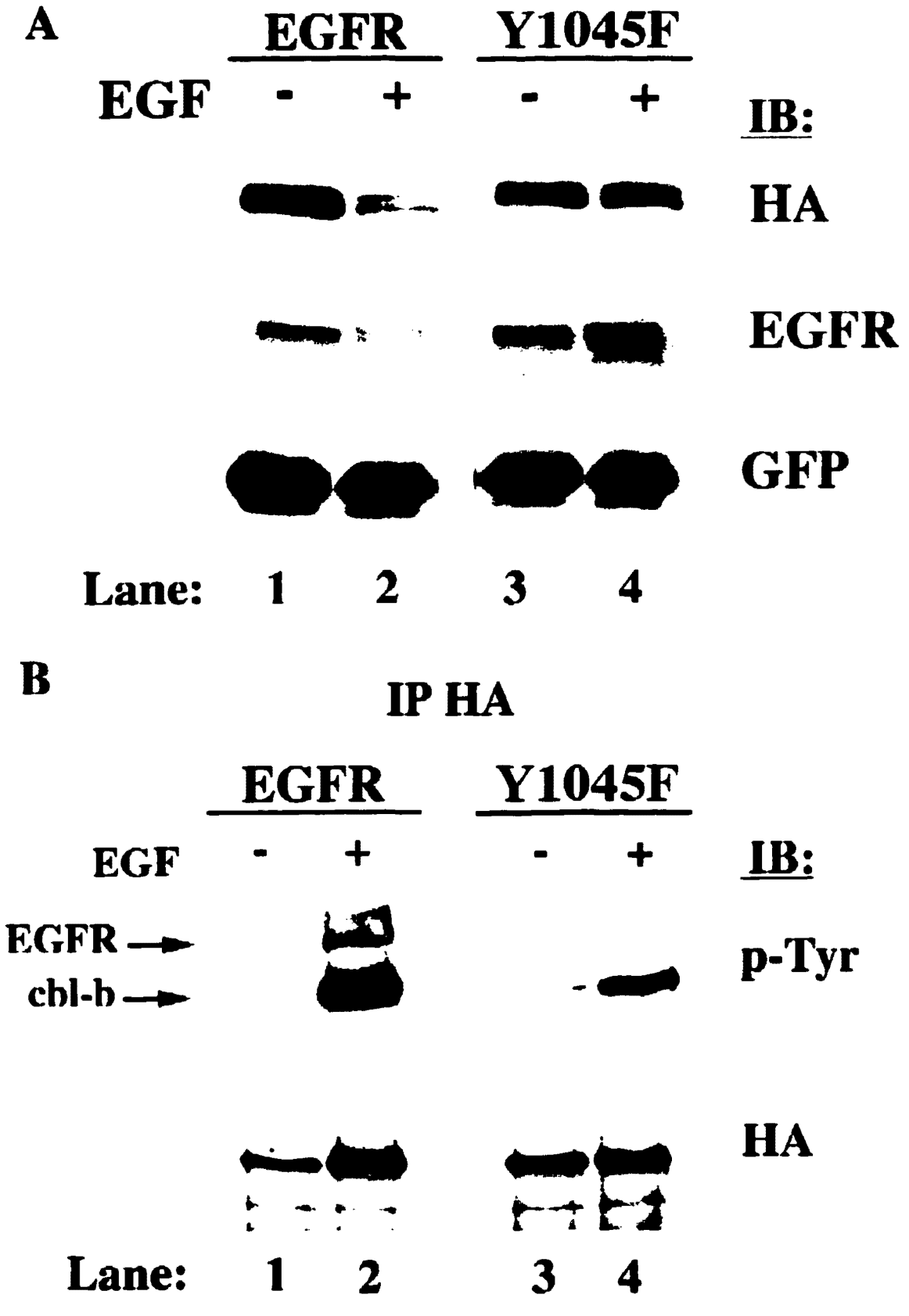

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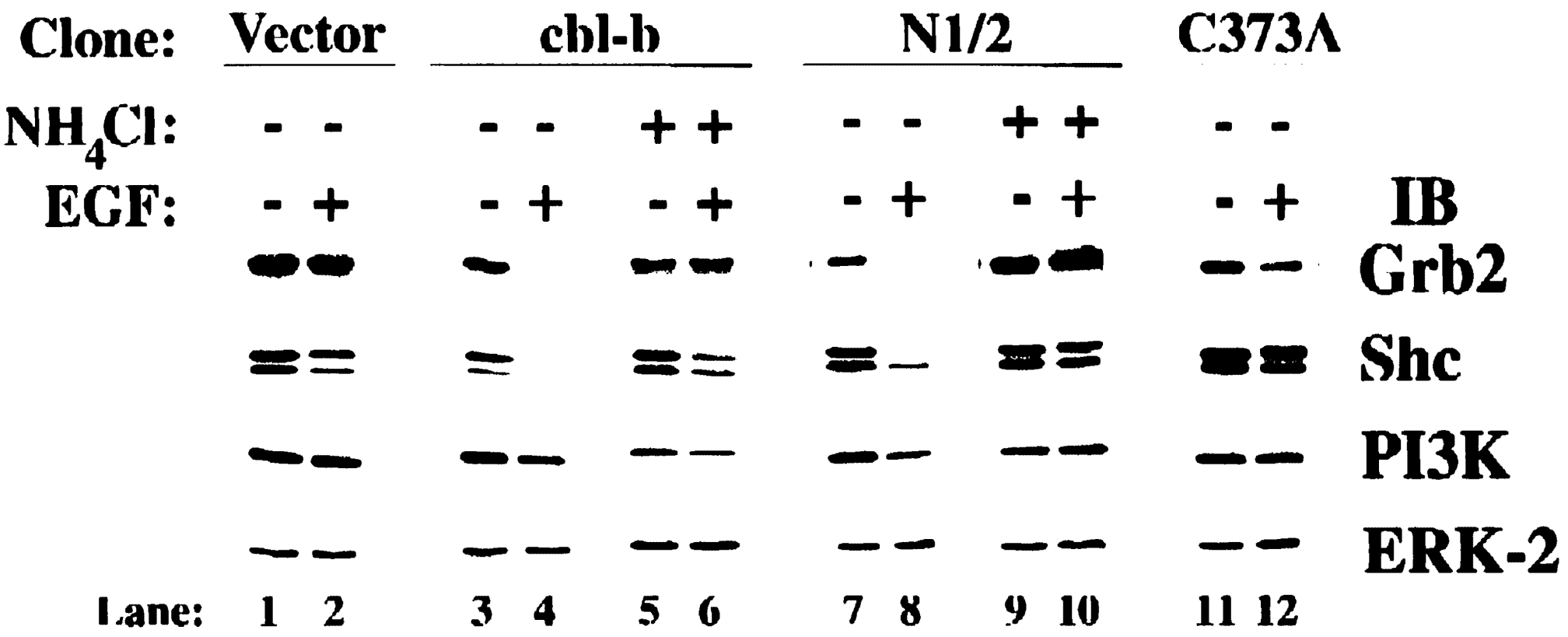
Fig. 7



Discussion

Attenuation of signaling by the EGFR is critical for normal cell growth and development [90]. The work presented in the papers which comprise this thesis describe one role for the cbl-b protein as a negative regulator of EGFR function.

To begin to characterize the newly identified cbl-b protein [91], biochemical studies of EGFR activation were carried out in transiently transfected 293T cells (described in Paper 1). cbl-b is rapidly phosphorylated upon stimulation of cells with EGF and is recruited to the activated EGFR (Paper 1, Figure 2). c-cbl is similarly phosphorylated and recruited upon EGFR activation and both cbl proteins compete with one another for binding to the activated EGFR (Paper 1, Figure 4). Like c-cbl, cbl-b associates with other signaling molecules. cbl-b is constitutively associated with GRB2 while the p85 subunit of PI-3K is recruited to cbl-b upon EGFR activation (Paper 1, Figure 9). To assess the ability of cbl-b to regulate EGFR function, cbl-b was over-expressed in the 32D/EGFR murine hematopoietic stem cell line. The 32D cell line is absolutely dependent on IL-3 for sustained growth and rapidly undergoes apoptosis in the absence of IL-3 [92]. 32D cells do not normally express EGFR or any other member of the erbB family and do not grow in EGF. When the EGFR is stably transfected into these cells, they can grow in either IL-3 or EGF [93]. Overexpression of cbl-b, but not c-cbl, inhibits EGFR dependent cell growth (Paper 1, Figure 5). 32D/EGFR cells over-expressing cbl-b grew as well as control cells in response to IL-3 indicating that the inhibition of EGFR function by cbl-b was specific to the EGFR signaling pathway. These data demonstrate a negative regulatory role for cbl-b in EGFR induced proliferation.

cbl-b was phosphorylated and recruited to the EGFR in the 32D/EGFR cell line. Further biochemical studies revealed that overexpression of cbl-b resulted in a shortened duration of activation of the MAPK, AKT and JNK downstream pathways (Paper 1, Figures 7 and 8). The binding of cbl-b to the activated EGFR and the inhibition of multiple downstream pathways initiated by EGFR signaling suggested that cbl-b functions at a step in the pathway near the receptor.

In the 32D/EGFR cell line, overexpression of cbl-b inhibited cell growth and resulted in an increase in the fraction of cells undergoing apoptosis. In the 32D/EGFR cell line removal of the mitogen (*i.e.* IL-3 or EGF) from the media also inhibits growth and causes an increase in apoptosis. Thus, these results are consistent with cbl-b inhibiting EGFR function. However, in some cells (*e.g.* MDA-MB-468 and MDA-MB-431) EGFR activation induces apoptosis. Therefore, it was possible that the inhibitory effects of cbl-b observed in the 32D/EGFR cell line were due to a change in signaling by the EGFR from growth stimulation to induction of apoptosis. These two possibilities could not be distinguished in the 32D/EGFR cell line since either mechanism (blockade of EGFR signaling or a change to apoptosis) would result in the same outcome of growth inhibition and increased apoptosis. To address this, stable clones overexpressing cbl-b were derived from the MDA-MB-468 human breast cancer cell line, which expresses high levels of the EGFR (described in the second paper). Stimulation of these cells with EGF induces apoptosis [32]. In this cell line if cbl-b functions by blocking EGFR signaling, then cells overexpressing cbl-b would not undergo EGF induced apoptosis (Figure 3). This would result in an increased viability of the cells in the presence of high concentrations of EGF. In contrast, if cbl-b enhanced EGFR induced apoptosis, then

clones overexpressing cbl-b would have an increase in EGF induced apoptosis resulting in a decreased viability at high concentrations of EGF.

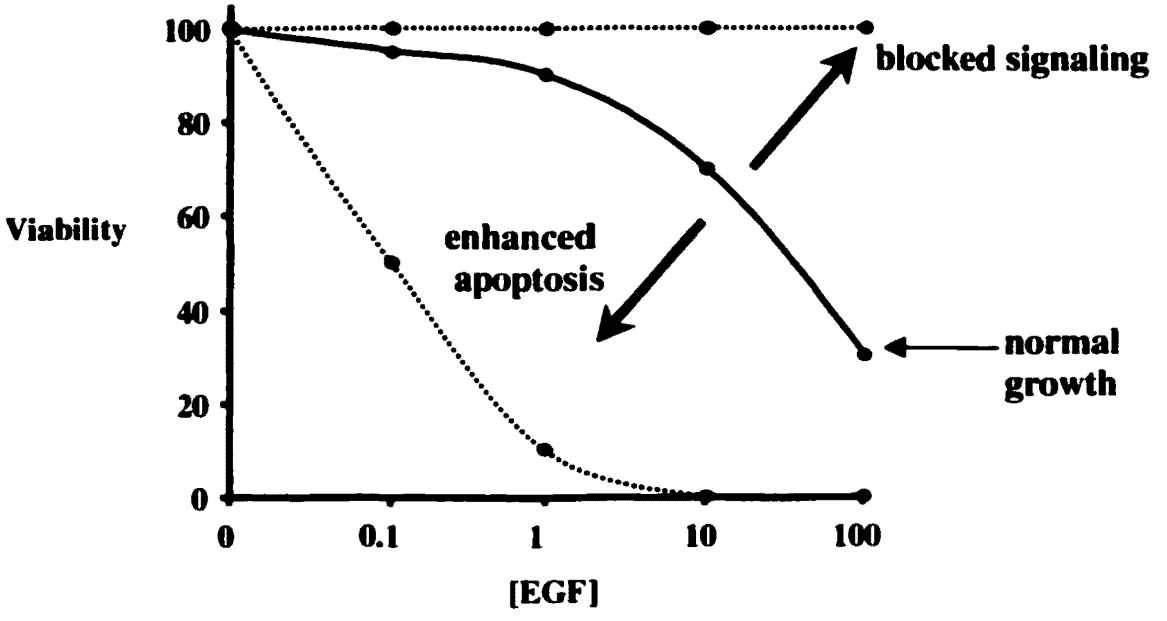


Figure 3: Hypothetical Growth Curve of cbl-b Stable Clones in the Presence of EGF. Solid black line represents actual growth curve of the parent MDA-MB-468 cell line in increasing concentrations of EGF after three days in culture. The decreased viability is due to EGF induced apoptosis. Dotted lines represent two possible outcomes of stable cbl-b overexpression in this cell line in increasing EGF concentrations after three days of growth. (See text.)

MDA-MB-468 cells overexpressing cbl-b showed no EGF induced apoptosis (Paper 2, Figure1A). These data confirmed that cbl-b inhibits EGFR signaling. Furthermore, cbl-b inhibits EGFR signaling in these two distinct systems regardless of the biological consequences of signaling.

It was difficult to study the biochemistry of EGFR signaling in the 32D/EGFR cells overexpressing cbl-b because the cells were undergoing apoptosis. During apoptosis many proteins, including the cbl proteins [94], are degraded in a caspase

dependent fashion. The MDA-MB-468 cells overexpressing cbl-b are not undergoing apoptosis. Therefore they are a better reagent for biochemical studies. Biochemical analysis of the EGFR over a 48 hour time course of EGF stimulation showed that the duration of activation of the EGFR, as measured by tyrosine phosphorylation, was shortened in the cbl-b overexpressing clones (Paper 2, Figure 3A). As seen in the 32D/EGFR cells, the duration of activation of multiple downstream signaling pathways (*e.g.* MAPK and STAT) in the cbl-b clones was shortened (Paper 2, Figure 3B and C).

The laboratory of Yosef Yarden reported that c-cbl overexpression enhanced the internalization and degradation of the activated EGFR through the covalent attachment of ubiquitin to the EGFR [95]. Ligand induced ubiquitin modification of several membrane receptors including PDGFR [96], growth hormone receptor [97], and the EGFR [98] has been documented. Ubiquitin conjugation of cellular proteins occurs through a series of enzymatic steps. First, free ubiquitin moieties become activated in an ATP dependent process and form a high-energy thioester bond between the C-terminal glycine of ubiquitin and a cysteine residue of the ubiquitin-activating enzyme, E1. The ubiquitin moiety is then transferred to a cysteine residue of a ubiquitin-conjugating, or E2, enzyme. Last, a ubiquitin-protein ligase, or E3, catalyzes the transfer of the ubiquitin from the E2 to the ε-NH_2 of an internal lysine residue of the target substrate generating an isopeptide bond. The E3 determines the specificity of the target substrate. In succeeding rounds of the reaction a poly-ubiquitin chain is assembled on the substrate by adding ubiquitin to lysine residue 48 of the previous ubiquitin moiety. This poly-ubiquitin chain allows recognition and binding of the substrate to the ATP-dependent 26S proteasome, which

contains proteases that degrade the target substrate into small peptide fragments releasing free ubiquitin moieties for reuse [99].

cbl-b overexpression in the MDA-MB-468 clones enhanced ubiquitination and degradation of the EGFR (Paper 2, Figure 4). Furthermore, a specific proteasomal inhibitor, Lactacystin, could block both the biochemical and the functional effects of cbl-b overexpression in these cells (Paper 2, Figure 6). The observation that cbl-b was found to function in the same manner as previously reported for c-cbl suggested that ubiquitination of tyrosine kinases was a conserved function of this family of proteins. In collaboration with the laboratory of Yosef Yarden, we demonstrated that all three mammalian members of the cbl family could enhance ubiquitination to the activated EGFR in an *in vivo* assay. Furthermore, *in vitro* reconstitution experiments demonstrated directly that cbl proteins catalyze the transfer of ubiquitin to the EGFR (See Appendix 2) [100]. Thus, the above results show that cbl proteins are ubiquitin E3 ligases. The E3 ligase activity of cbl proteins requires an intact TKB domain (to allow binding of cbl protein to the EGFR) and an intact RING finger domain (for catalytic activity) of the cbl protein. Additionally, activation of the EGFR and phosphorylation of EGFR tyrosine residue 1045 are required for cbl binding and receptor ubiquitination.

Upon further study of EGFR downregulation in the MDA-MB-468 stable clones overexpressing cbl-b, we observed that cbl-b is degraded upon EGFR activation (described in Paper 3, Figure 1). cbl-b was degraded with the EGFR in a stochiometric fashion. Further analysis revealed that both cbl-b and c-cbl are degraded upon EGFR activation. Inhibition of proteasomal and lysosomal degradation blocks degradation of both the EGFR and cbl-b (Paper 3, Figure 3). Furthermore, EGFR activation results in

ubiquitination of both the EGFR and cbl-b (Paper 3, Figure 4). In addition, stable clones were selected using mutants of the cbl-b protein. In these clones we assayed the structural requirements of cbl-b degradation. Like downregulation of the EGFR, cbl-b requires an intact TKB domain and an intact RING finger domain. Using a mutant EGFR, we demonstrated that degradation of cbl-b requires binding to the activated EGFR (Paper 3, Figure 6). Furthermore, other proteins bound to the activated EGFR complex are also degraded in MDA-MB-468 cells overexpressing cbl-b (*i.e.* GRB2 and Shc) (Paper 3, Figure 7). Together, these data suggest that cbl-b regulates a coordinated degradation of the active EGFR signaling complex.

A detailed mechanism of the role of cbl proteins in the process of EGFR downregulation can be made based on the results described above. (See Figure 4.) Ligand binding, receptor dimerization and receptor activation cause tyrosine phosphorylation of the receptor and activation of downstream Src kinases. These Src kinases are responsible for the rapid phosphorylation of cbl [101]. Furthermore, phosphorylation of the EGFR on tyrosine residue 1045 allows binding of a cbl protein to this site. Then the EGFR phosphorylates the cbl protein. cbl is believed to bind while the EGFR is still at the membrane or shortly after the receptor is internalized at the early endosome [95]. cbl catalyses the conjugation of ubiquitin to the EGFR, and itself. This process happens while the EGFR undergoes trafficking from the plasma membrane into endosomal vesicles. Finally, the EGFR, cbl, and other proteins bound to the activated EGFR complex undergo degradation which requires both proteasomal and lysosomal proteases.

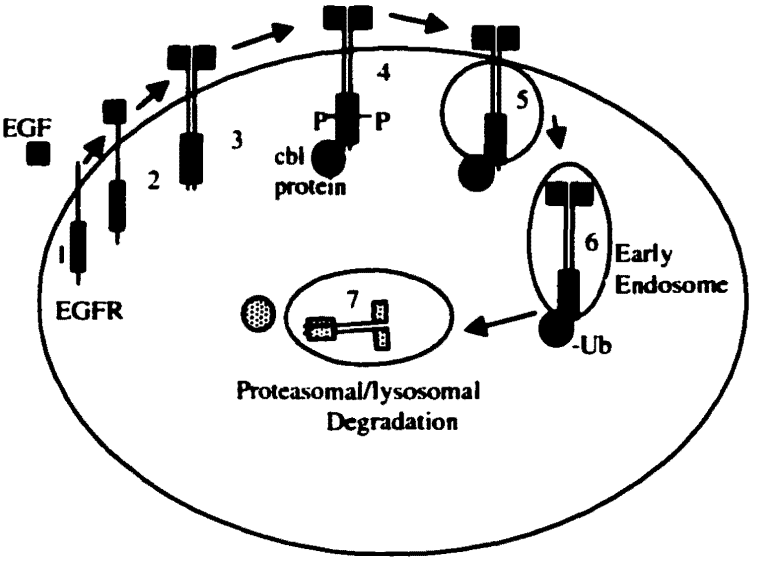


Figure 4: EGFR Downregulation by cbl Proteins. 1. Inactive EGFR monomer. 2. Ligand Binding. 3. Receptor dimerization. 4. Receptor activation, tyrosine phosphorylation, and complex formation. 5. Receptors entering clathrin coated pits. 6. Trafficking of receptors to early endosome and ubiquitination. 7. Ubiquitinated complex degradation.

One important aspect of this model which remains to be elucidated is the role of the proteasome and the lysosome in EGFR degradation. Experiments using protease inhibitors have demonstrated clearly that both proteasomal and lysosomal proteases are required for degradation of the EGFR, cbl-b, and other proteins of the EGFR signaling complex (Paper 3, Figures 3 and 7). However, it is unclear how the proteasome and lysosome cooperate to degrade the EGFR complex. The interaction between these two pathways is complex. For example, upon ligand binding the human Growth Hormone Receptor is internalized, ubiquitinated, and degraded in a fashion that depends on both the proteasome and lysosome just as for the EGFR [102]. Inhibition of either the proteasome or lysosome blocks growth hormone receptor degradation [103]. While the growth hormone receptor is thought to be degraded in the lysosome, inhibition of the

proteasome prevents internalization of the receptor suggesting that ubiquitnation and proteasomal degradation play a role in the trafficking. However, mutation of all of the C-terminal lysines of the growth hormone receptor (which prevents ubiquitination) does not interfere with its internalization or its degradation [104]. From these complex results, it is likely that other proteins are involved in the downregulation of activated growth factor receptors. For example, one interpretation of these results is that a protein other than the growth hormone receptor must be ubiquitinated and degraded for the receptor to be internalized and degraded. Whether the complex interactions between the proteasome and lysosome described for growth hormone receptor downregulation are also true for EGFR degradation remain to be seen.

cbl proteins are phosphorylated in response to activation of many different receptors and interact with a variety of receptors and signaling molecules [101]. In addition to the EGFR, cbl proteins have been shown to downregulate other receptor (*e.g.* CSFR, PDGFR) [105, 106] and non-receptor tyrosine kinases (*e.g.* Fyn) [107]. It is likely, therefore, that cbl proteins are involved in the negative regulation of a wide variety of signaling pathways.

An important question that requires further study is the specific function of each of the three mammalian cbl proteins. The conservation of the TKB and RING finger domains suggests that they all are likely to serve as E3 ligases for tyrosine kinases. Our studies suggest that there are significant differences between cbl-b and c-cbl. In the 32D/EGFR cell line, c-cbl, in contrast to cbl-b, did not inhibit EGF induced growth (Paper 1, Figure 5). In addition, c-cbl did not shorten the duration of activation of downstream signaling pathways as was seen with cbl-b (Paper 1, Figures 7 and 8). These

results are surprising since EGF induces c-cbl phosphorylation and recruitment to the EGFR in a variety of transient transfection experiments [108, 109]. Furthermore, c-cbl can enhance ubiquitination and degradation of the activated EGFR in transiently transfected cells [95, 110]. The functional and biochemical differences observed in the 32D/EGFR cells between cbl-b and cbl could result if cbl-b has a higher affinity for binding to and ubiquitinating the EGFR. The levels of the transfected proteins in the stable 32D/EGFR clones is significantly lower than those attained in the transiently transfected cells. Thus the high levels of protein generated in transiently transfected cells could overcome relative differences in affinities. Alternatively, other unidentified proteins may be required for the E3 activity of cbl proteins and these may be specific to the individual cbl proteins. In this case, the 32D/EGFR cells may express only the cbl-b specific cofactors and not the c-cbl cofactor.

In the MDA-MB-468 cells, we were unable to generate clones stably overexpressing c-cbl. This suggests that overexpression of c-cbl is toxic to these cells while overexpression of cbl-b is not. While the exact reason that c-cbl clones do not grow is difficult to demonstrate, this highlights another difference between these two related proteins. There are other biochemical examples of differences between c-cbl and cbl-b. For example, cbl-b activates the ZAP 70 kinase and NFAT in T-lymphocytes [83] while c-cbl inhibits their activation [111]. In addition, c-cbl associates with the 14-3-3 protein upon T-cell receptor activation while cbl-b does not [112]. Studies in mice with homozygous deletions of either c-cbl or cbl-b have revealed physiologic differences between these two cbl proteins [113-116]. Mice lacking c-cbl have altered thymic selection of T-cells, enhanced activation of T-cell receptor signaling pathways in mitogen

activated thymocytes, and increased mitogen induced thymocyte proliferation [113]. In contrast, mice lacking cbl-b have normal thymocyte development. Also these mice have increased activation of the CD28 costimulatory pathway in mitogen activated mature T-cells, increased proliferation in mitogen activated T-cells, and develop autoimmunity [115]. Overall, there is clear biochemical and physiological evidence that c-cbl and cbl-b have distinct functional roles, in spite of their structural similarity. More work is needed to define the precise role of each of the three mammalian cbl proteins and to clarify the molecular basis for these differences.

In summary, the work presented in this thesis has identified a role for cbl-b as a negative regulator of EGFR signaling and has allowed for the development of a molecular model of cbl-b function. It is known that cbl proteins are phosphorylated in response to activation of many different receptors. Furthermore, cbl proteins interact with a variety of receptors and signaling molecules [80]. Whether cbl proteins act as E3 ligases for these other signaling pathways remains to be elucidated. The physiologic relevance of the cbl proteins also remains to be completely determined. Data from the cbl-b knockout mouse suggest that the loss of cbl-b function could result in autoimmune disease [115]. Indeed, recent work has suggested that lymphocytes from patients with Systemic Lupus Erythematosus fail to activate cbl proteins in response to mitogen stimulation [117]. Additionally, a role for cbl proteins in malignant transformation is suggested by the oncogenic form of cbl found in the 70Z murine lymphoma cells [118]. Clearly, future studies are likely to yield interesting and important information about the cbl proteins.

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Appendix 1

Abbreviations:

EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
EGFRvIII	Epidermal Growth Factor Variant Three
GAP	GTPase Activating Protein
GM-CSF	Granulocyte Macrophage-Colony-Stimulating Factor
GRB2	Growth factor Receptor Binding protein 2
IL-3	Interleukin-3
JNK	c-Jun N-Terminal Kinase
MAPK	Mitogen Activated Protein Kinase
PDGF	Platelet Derived Growth Factor
PLCγ	Phospholipase-C gamma
PRL	Prolactin
SDS-PAGE	Sodium-Dodecyl-Sulfate Polyacrylamide Gel Electrophoresis
SH2	Src Homology 2 domain
SH3	Src Homolgy 3 domain
SOS	Son Of Sevenless protein
STAT	Signal Transducer and Activator of Transcription
TGF-α	Tumor Growth Factor – alpha
TKB	Tyrosine Kinase Binding domain

Appendix 2

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Ubiquitin Ligase Activity and Tyrosine Phosphorylation Underlie Suppression of Growth Factor Signaling by c-Cbl/Sli-1

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Summary

Receptor desensitization is accomplished by accelerated endocytosis and degradation of ligand–receptor complexes. An in vitro reconstituted system indicates that Cbl adaptor proteins directly control downregulation of the receptor for the epidermal growth factor (EGFR) by recruiting ubiquitin-activating and -conjugating enzymes. We infer a sequential process initiated by autophosphorylation of EGFR at a previously identified lysosome-targeting motif that subsequently recruits Cbl. This is followed by tyrosine phosphorylation of c-Cbl at a site flanking its RING finger, which enables receptor ubiquitination and degradation. Whereas all three members of the Cbl family can enhance ubiquitination, two oncogenic Cbl variants, whose RING fingers are defective and phosphorylation sites are missing, are unable to desensitize EGFR. Our study identifies Cbl proteins as components of the ubiquitin ligation machinery and implies that they similarly suppress many other signaling pathways.

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Introduction

Cellular activation by a large variety of extracellular stimuli is generally followed by transient refractoriness to the same stimulus. One of the better characterized mechanisms of homologous desensitization involves a rapid decrease in the number of the respective cell surface receptors through accelerated endocytosis (Schmid, 1997). This process is termed "downregulation," and it is shared by receptors for growth factors, lymphokines, antigens, and immunoglobulins. The biochemical mechanism underlying receptor downregulation are relatively well understood in the case of tyrosine kinase receptors (RTKs) for growth factors, such as the epidermal growth factor (EGF) and the platelet-derived growth factor (PDGF) (Sorkin and Waters, 1993). Ligand binding to surface receptors activates their efficient internalization via clathrin-coated pits that invaginate to form coated vesicles. Unlike receptors for nutrients (e.g., the transferrin receptor), a significant pool of activated RTKs escapes recycling to the cell surface and is sorted to the lysosome degradation pathway. Sorting of internalized receptors to this pathway is regulated, at least in part, by the intrinsic tyrosine kinase activity of the receptor (French et al., 1994), but it remained poorly understood until very recently. An initial clue has been provided by the invertebrate form of the EGF receptor (EGFR): vulval development in *C. elegans* and development of the R7 photoreceptor in *Drosophila*, two processes that are critically controlled by the EGFR system, are negatively regulated by an adaptor protein called Sli-1/Cbl (Yoon et al., 1995; Meisner et al., 1997).

Sli-1 is an ortholog of the v-cbl transforming gene of the CAS NS-1 retrovirus known to induce pre–B lymphoma and myeloid leukemia (Langdon et al., 1989). Early and prominent tyrosine phosphorylation of the ubiquitously expressed 120 kDa c-Cbl protein was demonstrated in T and B cells stimulated with antigens and in other cell types upon activation by growth factors (e.g., EGF, PDGF, fibroblasts growth factor, colony-stimulating factor, nerve growth factor, prolactin, insulin, and the stem cell factor), cytokines (e.g., interleukin [IL]-2, IL-3, IL-4, interferon-α, erythropoietin, and thrombospondin), fibronectin, and immunoglobulins (for review, see Thien and Langdon, 1998). Overexpression of c-Cbl and transfection of dominant oncogenic mutants uncovered a negative regulatory role of the p120 c-Cbl protein in mammalian cells: not only the EGFR (Levkowitz et al., 1998) and the receptor for PDGF (Miyake et al., 1998) are downregulated upon c-Cbl overexpression, but the function of several immune receptor-coupled tyrosine kinases (e.g., Syk [Ota and Samelson, 1997]) is also suppressed by c-Cbl. Understanding the mechanism by which Cbl proteins downregulate signaling is hampered by the fact that these proteins possess no known catalytic activity. Nevertheless, the functions of several c-Cbl's structural domains have been elucidated. The N-terminal half of Cbl contains a unique Src homology 2 (SH2) domain, which mediates binding to tyrosine-phosphorylated receptors (Meng et al., 1999).

The C-terminal half carries a long proline-rich domain and several tyrosine phosphorylation sites that mediate constitutive and inducible interactions with kinases (e.g., Src) and guanine nucleotide exchange factors (e.g., Vav, C3G, and SOS). A centrally located C_3HC_4-type RING finger (RF) domain separates the two adaptor domains of c-Cbl. A clue to its role may be provided by the fact that the two other family members, Cbl-b and Cbl-3 (Keane et al., 1995, 1999), carry an intact RF. However, this domain is interrupted in the two oncogenic forms of c-Cbl, namely v-Cbl and 70Z-Cbl, an oncoprotein found in a pre-B lymphoma cell line (Andoniou et al., 1994). Consistent with the importance of the RF, mutagenesis of its first cysteine abrogated the suppressive activity (Waterman et al., 1999).

Several recent studies implicate RF domains of other proteins in recruitment of the ubiquitin–proteasome degradation pathways (Tyers and Willems, 1999). The possibility that Cbl proteins may regulate protein ubiquitination through their RF is supported by several studies demonstrating that Cbl overexpression elevates ubiquitination of the receptors for EGF (Levkowitz et al., 1998), PDGF (Miyake et al., 1998), and CSF-1 (Lee et al., 1999). The present study addressed the molecular mechanism underlying Cbl-induced ubiquitination and degradation of RTKs. Assuming a direct role of c-Cbl, we first reconstituted a cell-free system that processes ubiquitination and degradation of isolated EGFRs. Using this reconstituted system, we learned that Cbl is a component of the ubiquitin ligation machinery specific for ligand-activated RTKs. Mutagenesis of the major partners, Cbl and EGFR, unexpectedly revealed the necessity of two tyrosine phosphorylation events for productive ubiquitination and subsequent receptor sorting to degradation: both EGFR and c-Cbl must undergo phosphorylation on specific sites. This requirement represents the first example of the ability of tyrosine phosphorylation to tag proteins for proteasomal degradation.

Results

Ubiquitination of an Isolated EGFR and Its Degradation In Vitro Are Directly Regulated by Cbl

c-Cbl can accelerate degradation of EGFR in living cells by increasing receptor ubiquitination (Levkowitz et al., 1998; Waterman et al., 1999). This function is not limited to c-Cbl; the two recently described homologs of c-Cbl, namely Cbl-b (Keane et al., 1995) and Cbl-3 (Keane et al., 1999), are also active. Overexpression of Cbl-b or Cbl-3 in Chinese hamster ovary (CHO) cells led to an accelerated removal from the cell surface of a cooverexpressed EGFR (Figure 1A and data not shown). Concomitant with accelerated endocytosis, the receptor underwent enhanced ubiquitination and degradation (Figure 1B). Interestingly, an alternatively spliced short variant of Cbl-3 (Cbl-3S), whose SH2 domain is defective, did not affect ubiquitination of EGFR. On the other hand, the two known oncogenic variants of Cbl, v-Cbl and 70Z-Cbl (Langdon et al., 1989), inhibit rather than stimulate the rate of receptor downregulation (Figure 1 and data not shown). Taken together, the results presented in Figure 1 indicate that all three mammalian Cbl proteins are involved in desensitization of EGFR.

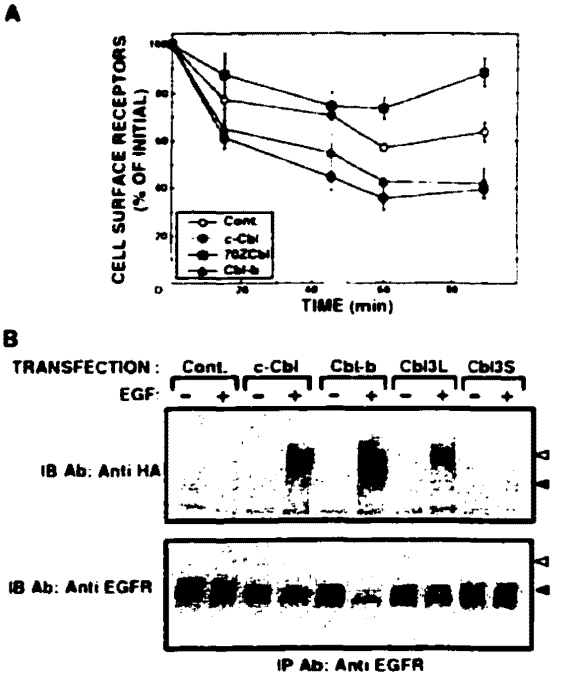

Figure 1. Downregulation and Ubiquitination of EGFR in Living Cells

(A) CHO cells were cotransfected with an EGFR expression vector, along with plasmids encoding the indicated Cbl proteins or an empty vector (Cont.). Thereafter, cultures were incubated at 37°C with EGF (100 ng/ml) for the indicated periods of time. Thereafter, cell-bound ligand was removed, and the level of surface receptors was determined in triplicates by binding of a radiolabeled EGF at 4°C.

(B) CHO cells were cotransfected with an EGFR expression vector and a plasmid encoding a hemagglutinin (HA)–tagged ubiquitin, along with the indicated c-Cbl, Cbl-b, and Cbl-3 plasmids. Both a short form of Cbl-3 (Cbl-3S) and a long form (Cbl-3L) were tested. Monolayers were incubated for 15 min at 37°C with or without EGF (at 100 ng/ml), and cell lysates subjected to immunoprecipitation (IP) and immunoblotting (IB) with the indicated antibodies. Closed and open arrowheads, respectively, mark the locations of the unmodified and the ubiquitinated forms of EGFR.

To better understand the function of Cbl proteins in receptor ubiquitination and degradation, we aimed at constructing an in vitro ubiquitination and degradation system by using recombinant components. A previous attempt to reconstitute EGFR ubiquitination has identified rabbit reticulocyte lysate as a useful source of modifying enzymes (Mori et al., 1997), and our initial utilization of a similar system has attributed an essential role to c-Cbl (Waterman et al., 1999). Two versions of the in vitro ubiquitination system are presented in Figure 2A. Both systems use an immunopurified EGFR, a rabbit reticulocyte lysate, ATP, and ubiquitin. Ligation of ubiquitin to EGFR was followed by either labeling of the receptor (with a radioactive phosphate) or by using a radioactive ubiquitin. As we previously reported, bacterially expressed c-Cbl fused to glutathione S-transferase (GST–Cbl) enabled receptor ubiquitination (Figure 2A). Another SH2 protein, namely the Shc protein, was inactive. Control reactions performed in the presence of a large excess of an unlabeled ubiquitin or three other EGFR-interacting proteins, the p85 regulatory subunit of

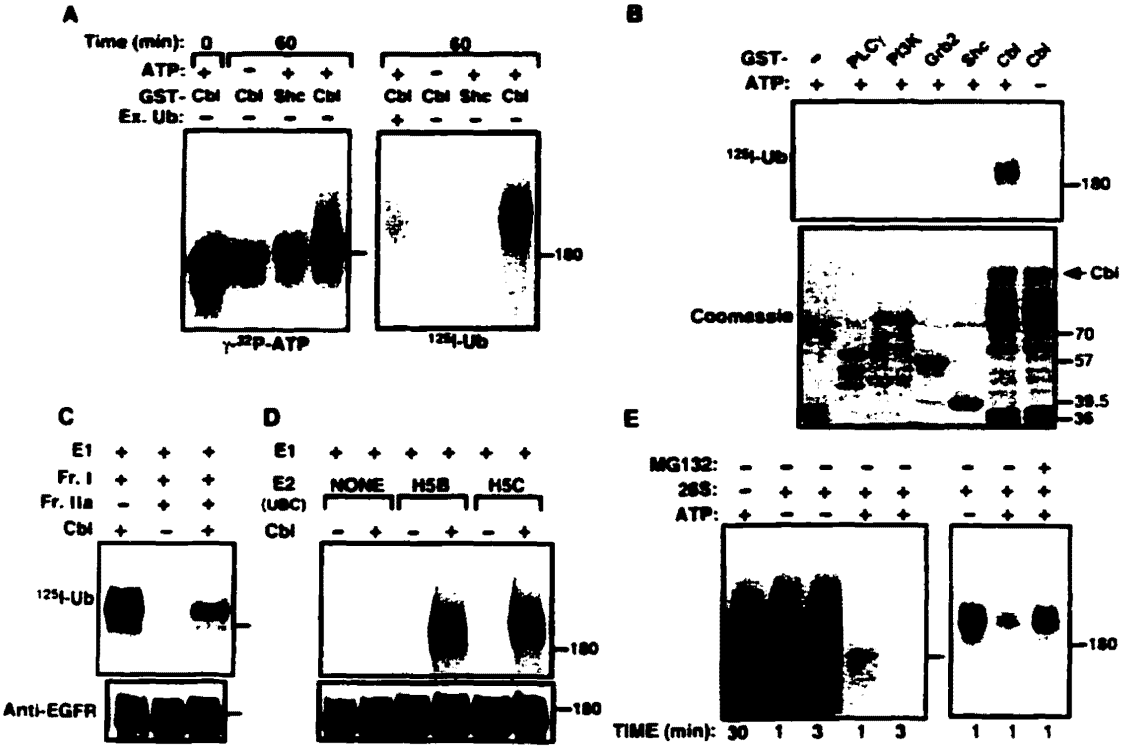

Figure 2. In Vitro Reconstitution of EGFR Ubiquitination and Degradation

(A) (Left) EGFR immunocomplexes were prelabeled with ^{32}P and subjected to an in vitro ubiquitination reaction, in the presence of rabbit reticulocyte lysate and the indicated GST fusion proteins, as detailed in the Experimental Procedures. (Right) An in vitro ubiquitination reaction was similarly performed except that an unlabeled EGFR was used and the reaction was performed in the presence of a radiolabeled ubiquitin (^{125}I-Ub). Reactions were carried out in the absence or presence of a 100-fold excess of an unlabeled ubiquitin (Ex. Ub).

(B) In vitro ubiquitination was performed with a radiolabeled ubiquitin in the presence of one of the following GST fusion proteins: the SH2 domains of PI3K, PLCγ-1, and Shc or full-length Grb-2 and c-Cbl proteins. For control, we used a GST protein alone or omitted ATP, as indicated. The bottom panel shows a stained gel with the respective fusion protein preparations.

(C) In vitro ubiquitination was performed as in (B), except that a purified E1 enzyme and the indicated chromatographic fractions of reticulocyte lysates replaced the crude whole lysate. Gel-resolved proteins were transferred to filters, which were first autoradiographed (upper panel, ^{125}I-Ub) and then immunoblotted with anti-EGFR antibodies.

(D) Reactions were performed as in (C) except that recombinant E1 (from insect cells) and the indicated E2 proteins (from bacteria) were used.

(E) Purified EGFR was labeled with ^{125}I-ubiquitin, washed, and incubated at 37°C for the indicated time intervals in the absence or presence of a purified 26S proteasome preparation, ATP, or MG132, as indicated.

phosphoinositide 3-kinase (PI3K), phospholipase Cγ-1 (PLCγ), and Grb-2, confirmed saturability and specificity to Cbl (Figures 2A and 2B).

Although these results indicate that c-Cbl is involved in covalent attachment of ubiquitin to EGFR, they leave open the exact role it plays in the underlying three-step enzymatic reaction. Adenylation of ubiquitin by the ubiquitous E1 enzyme is followed by transfer of the activated molecule to one of several types of E2 enzymes. These relay ubiquitin either directly or indirectly through an E3 ubiquitin ligase, to the target protein (for review, see Hershko and Ciechanover, 1998). Chromatographic fractionation of whole reticulocyte lysates can separate the major E2 activity (fraction I) from several E3-like activities (fraction IIa) (Abu Hatoum et al., 1998). When tested for ubiquitination of EGFR in the presence of a purified E1, the combination of the two fractions was inactive, but c-Cbl could reconstitute the activity in the presence of E1 and fraction I (Figure 2C). Evidently, none of the fraction IIa–enriched E3s could replace c-Cbl. Out of five recombinant E2s that we tested, only two enzymes, UBC-H5B and UBC-H5C, were active; UBC-H5A, UBC-H7, and UBC-3/CDC-34 were inactive, as was fraction IIa, which may contain some E2 activity (Figure 2D and data not shown). These results imply that c-Cbl mediates transfer of ubiquitin from a specific E2 to EGFR. Once tagged by ubiquitin, EGFR is destined to intracellular degradation that can be partially inhibited by the proteasomal inhibitor MG132 (Levkowitz et al., 1998). We reconstituted this activity by incubating an in vitro ubiquitinated EGFR with a purified 26S proteasome preparation (Figure 2E). As expected, receptor proteolysis was rapid, ATP dependent, and sensitive to MG132. In conclusion, because c-Cbl can support ubiquitination of EGFR in vitro in the presence of isolated recombinant E1 and E2, and the modified receptor is processed by the proteasome, c-Cbl probably acts as a ubiquitin ligase or a ligase-ancillary protein.

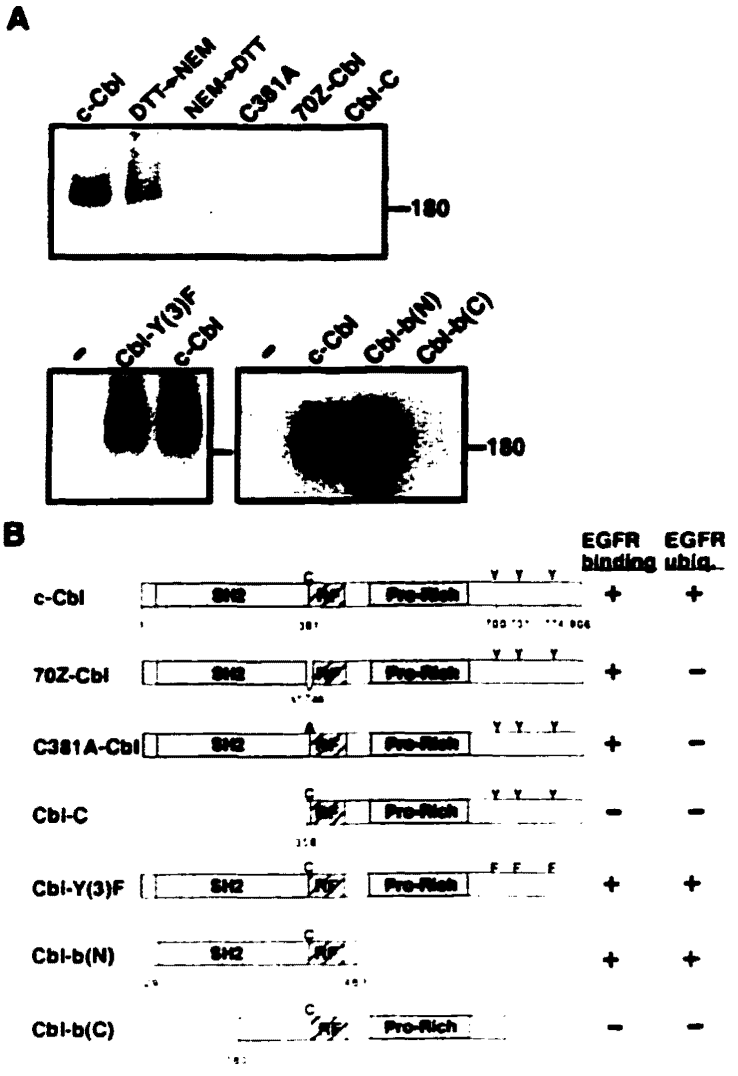

Figure 3. Structural Requirements of the Cbl-Induced Ubiquitin Conjugation Activity

(A) In vitro ubiquitination reactions were performed with recombinant E1 and E2 (UBC-H5B). To test the effect of alkylation, c-Cbl was treated for 10 min at 22°C with NEM and then with DTT (each at 10 mM) or this sequence was reversed.

(B) The domain structures and in vitro activities of Cbl proteins are shown, and residue numbers are indicated. Boxes correspond to the SH2 domain, a RING finger domain (RF), and a proline-rich domain. Three previously identified tyrosine phosphorylation sites are indicated by Y letters, and a mutant in which all three sites were mutated to phenylalanine (Cbl-3YF) is depicted. An internal deletion of 17 amino acids (Δ17 aa) in 70Z-Cbl is also marked. The abilities of Cbl proteins to bind EGFR in vitro and induce its ubiquitination are summarized in a table.

The SH2 and RING Finger Domains of c-Cbl Are Necessary and Sufficient for Ubiquitin Ligation to EGFR

The structural determinants of c-Cbl necessary for its ubiquitin-conjugating activity were addressed by testing in vitro a series of GST–Cbl proteins. As expected, the oncogenic 70Z-Cbl protein was inactive in vitro (Figure 3A), although it differed from c-Cbl by only a short stretch of amino acids overlapping part of the RF (see scheme in Figure 3B). On the other hand, an amino-terminal portion of Cbl-b [Cbl-b(N)] retained EGFR binding, and it reconstituted ubiquitination in vitro, indicating that the proline-rich domain and the carboxyl terminus are dispensable for ubiquitin ligation. Lack of direct involvement of the three C-terminal tyrosine phosphorylation sites (Y700, Y731, and Y774 [Feshchenko et al., 1998]) was implied by the ability of a protein mutated at these specific sites, Cbl-Y(3)F, to reconstitute ubiquitination in vitro (Figure 3A). A partially overlapping part of Cbl-b [denoted Cbl-b(C)], as well as an N-terminal truncated form of c-Cbl (Cbl-C), were inactive, probably because their SH2 domains were defective. These observations implied that a combination of an intact SH2 domain and an uninterrupted RF is sufficient for EGFR modification. We have previously reported that a c-Cbl mutant, whose most N-terminal cysteine of the RF (residue 381) was replaced by an alanine, displays defective functions in living cells (Waterman et al., 1999). We verified lack of activity of the cysteine-to-alanine mutant (C381A-Cbl) in a ubiquitination reaction that uses recombinant E1 and E2 proteins (Figure 3A). This observation is consistent with the notion that the redox potential of many other RING fingers is essential for their activity (Saurin et al., 1996). Indeed, alkylation of c-Cbl with N-ethylmaleimide (NEM) completely abolished its activity, but pretreatment with dithiothreitol (DTT) protected the protein (Figure 3A). A summary of receptor ubiquitination by various Cbl mutants and their ability to bind EGFR in vitro is presented in Figure 3B. As expected, an intact SH2 domain is essential for receptor binding,

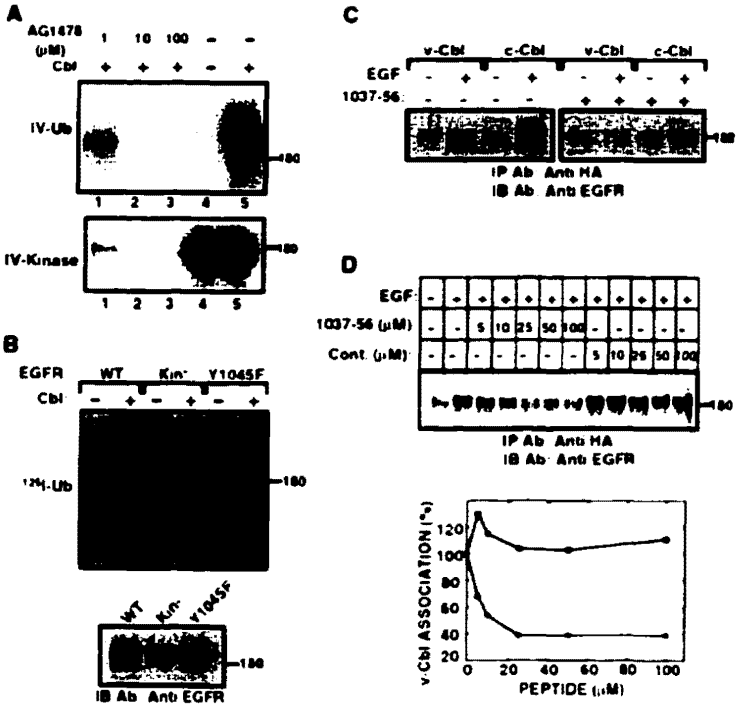

Figure 4. Phosphorylation at Tyrosine 1045 of EGFR Is Necessary for c-Cbl Binding and for Receptor Ubiquitination In Vitro

(A) EGFR immunoprecipitates were washed and pretreated for 20 min at 22°C with the indicated concentrations of AG1478. Thereafter, an in vitro ubiquitination assay was performed in the presence or absence of a recombinant GST–Cbl fusion protein. The bottom panel presents the results of an in vitro kinase assay performed with the corresponding samples in the presence of γ-^{32}P-labeled ATP.

(B) Wild type (WT), kinase-defective (Kin−), and the Y1045F mutant of EGFR were transiently overexpressed in HEK-293 cells. Receptor immunoprecipitates were subjected to an in vitro ubiquitination assay with a radiolabeled ubiquitin in the presence or absence of GST–Cbl, as indicated. The lower panel shows Western blotting of aliquots of the respective whole-cell lysates.

(C) HEK-293T cells were transfected with vectors directing expression of wild-type EGFR together with plasmids encoding HA-tagged c-Cbl or v-Cbl. Cells were treated for 10 min at 37°C without or with EGF (at 100 ng/ml) 48 hr later. Thereafter, whole-cell lysates were prepared and subjected to immunoprecipitation (IP) with an anti-HA antibody. Where indicated, immunoprecipitation was performed in the presence of a synthetic phosphopeptide (100 μM) corresponding to amino acids 1037–1056 of EGFR. Immunoprecipitates were analyzed by immunoblotting (IB) with anti-EGFR antibodies.

(D) Whole lysates derived from HEK-293T cells transiently expressing EGFR and v-Cbl were mixed with increasing concentrations of the indicated phosphopeptide prior to immunoprecipitation of v-Cbl by using anti-HA antibodies. Immunocomplexes were resolved by gel electrophoresis, transferred to nitrocellulose filters, and the filters blotted with anti-EGFR antibodies. The lower panel shows the densitometry results of the competition assay. Open symbols refer to signals obtained with the control phosphopeptide, and closed circles represent the results obtained with the phosphopeptide flanking tyrosine 1045. Signals were normalized to Cbl binding to EGFR in the absence of peptide.

and it serves as a prerequisite for EGFR ubiquitination. On the other hand, an intact RF is essential for receptor ubiquitination, but the whole C-terminal half of c-Cbl is not necessary for either activity.

Phosphorylation at Tyrosine 1045 of EGFR Creates a Major Docking Site for c-Cbl

Although tyrosine phosphorylation of EGFR is essential for stable association of the receptor with c-Cbl, indirect interactions that involve adaptor proteins such as Grb-2 may also link the two proteins (Levkowitz et al., 1996). The availability of a functional in vitro assay of c-Cbl allowed us to test the role of the two types of interactions and also identify the Cbl's docking site. To test the possibility that receptor phosphorylation is necessary for its modification by ubiquitin, we made use of a highly specific inhibitor of the kinase, AG1478 (Gazit et al., 1996). Relatively low concentrations of the antagonist inhibited autophosphorylation of EGFR in a dose-dependent manner (Figure 4A, lower panel), and a parallel decrease in receptor ubiquitination was observed (Figure 4A, upper panel). We then tested the prediction that a kinase-defective EGFR will undergo no ubiquitination in vitro. Indeed, when a wild-type receptor and a kinase-defective mutant were each transiently expressed in HEK-293 human cells and their ubiquitination tested in vitro, we found that only the wild-type form underwent ubiquitination (Figure 4B).

The association between receptor autophosphorylation and ubiquitination in vitro implied that a specific tyrosine autophosphorylation site of EGFR serves as a docking site for c-Cbl. To identify a Cbl-specific docking site, we made use of a series of receptor proteins mutated at individual tyrosines of the carboxyl terminus. Because none of six C-terminal tyrosine residues is necessary for ubiquitination of EGFR in living cells (Levkowitz et al., 1998), we concentrated on the remaining two tyrosine residues (tyrosines 1045 and 1101). The respective mutants were expressed in HEK-293 cells, and the receptors were tested in a ubiquitination assay. This assay revealed that one of the mutants, a receptor whose tyrosine 1045 was changed into a phenylalanine, lost the ability to undergo ubiquitination (Figure 4B). Control experiments verified expression of the mutant receptors and their ability to undergo autophosphorylation (Figure 4B, lower panel, and data not shown). Consistent with the identification of tyrosine 1045, the C-terminally flanking amino acid sequence conforms to the consensus Cbl docking site as characterized with the ZAP-70 tyrosine kinase (Tyrosine-X-X-X-Proline [Meng et al., 1999]). To directly test the docking ability of tyrosine 1045 and the surrounding amino acid sequence, we synthesized the corresponding tyrosine-phosphorylated peptide. The effect of the peptide on the association between EGFR and two Cbl proteins, a wild-type c-Cbl and v-Cbl, was tested in vitro. Because

v-Cbl contains only the SH2 domain, indirect interactions with EGFR, which are mediated by the proline-rich and the C-terminal tyrosines of c-Cbl, are excluded. Figure 4C depicts the results of the association experiment. Evidently, the peptide only slightly affected binding of c-Cbl to EGFR, but it almost completely abolished the interaction with the shorter viral form of Cbl. The specificity of the inhibitory effect was then tested by comparing increasing concentrations of the EGFR phosphopeptide with an unrelated phosphotyrosine peptide of similar length (Figure 4D). Whereas the specific phosphopeptide displaced EGFR from v-Cbl, the control peptide was ineffective. In conclusion, c-Cbl interacts with EGFR in an inducible manner primarily through an autophosphorylated tyrosine 1045 of the receptor.

The c-Cbl's Docking Site of EGFR Mediates Ligand-Induced Ubiquitination and Downregulation of the Receptor

Because tyrosine 1045 has not been previously identified as one of the five major sites of EGFR autophosphorylation, we predict it serves as a minor site. Indeed, Western blotting with antibodies to phosphotyrosine detected no significant difference between the content of phosphotyrosine in wild-type and in mutant (Y1045F) forms of EGFR (Figure 5A). However, in line with the results of in vitro assays, no basal interaction and only residual ligand-induced association between c-Cbl and the mutant form of EGFR was observed in living cells. Likewise, no up-smearing of EGFR, a characteristic of ubiquitination, was noted with the mutant receptor (Figure 5A). Despite its relatively weak interaction with c-Cbl, the mutant Y1045F receptor retained the ability to increase tyrosine phosphorylation of c-Cbl (Figure 5B). This observation is reminiscent of the ability of a mutant EGFR lacking the whole C-terminal tail to enhance Cbl phosphorylation (Levkowitz et al., 1998). Unlike the interrupted physical association with c-Cbl, mutagenesis of tyrosine 1045 did not interfere with the interaction with another substrate of the EGFR, namely Shc (Figure 5A).

Identification of the Cbl's docking site enabled us to study the role of Cbl–EGFR interactions in living cells. In line with the observation that an overexpressed c-Cbl enhances ubiquitination and degradation of the receptors for EGF (Levkowitz et al., 1998), the Cbl-defective mutant of EGFR (Y1045F) displayed an attenuated downregulation (Figure 5C). Nevertheless, residual ligand-induced downregulation of Y1045F was observed upon Cbl overexpression, indicating the existence of secondary mechanisms of Cbl recruitment to EGFR. The differences between the wild-type and mutant receptor forms were much more prominent when receptor degradation and ubiquitination were analyzed: EGF-induced degradation, as well as ubiquitination, of the mutant receptor in cells overexpressing c-Cbl was almost completely abolished (Figure 5D). Taken together, the results presented in Figure 5 indicate that tyrosine 1045 of EGFR acts as a major c-Cbl docking site, which mediates receptor degradation by enhancing ubiquitination.

Tyrosine Phosphorylation of c-Cbl at a Site Flanking the RING Finger Is Essential for an E3-like Ubiquitin Ligase Activity

Although prominent phosphorylation of c-Cbl has been reported in cells stimulated by a variety of ligands (Thien and Langdon, 1998), the possibility that Cbl phosphorylation regulates its activity has not been addressed before. Our in vitro assay of c-Cbl allowed us to examine this scenario. EGFR was isolated, and its autophosphorylation was allowed in vitro prior to inhibition of further phosphorylation by using a tyrphostin. Thereafter, c-Cbl was added and receptor ubiquitination tested. Consistent with a requirement for *trans*-phosphorylation of c-Cbl, we observed no receptor ubiquitination under these conditions (Figure 6A). Preincubation of EGFR with c-Cbl prior to adding the inhibitor was sufficient for ubiquitination, probably because *trans*-phosphorylation enabled Cbl activation. The use of two mutants of Cbl provided an initial mapping of the putative Cbl's phosphorylation site: a mutant whose three major C-terminal autophosphorylation sites were defected [Cbl-3(Y)F] and a truncated form of Cbl-b, denoted Cbl-b(N), also displayed dependence on prephosphorylation (Figure 6A). Thus, the putative Cbl's site of phosphorylation probably resides in the N-terminal half. To support this conclusion, we prephosphorylated both EGFR and the short form of Cbl-b and then mixed them under conditions that completely block further phosphorylation. As predicted, EGFR ubiquitination could be recovered even when tyrosine phosphorylation was completely inhibited, but prephosphorylation of both EGFR and c-Cbl was absolutely essential (Figure 6B). Incomplete recovery is likely due to the low efficiency of Cbl phosphorylation in vitro (Figure 6B, right panel).

To map the site of Cbl, whose phosphorylation is essential for its ubiquitin ligase activity, we concentrated on N-terminal tyrosine residues that are relatively conserved in the Cbl family. Mutagenesis of tyrosines 92, 291, 274, 307, 337, and 368 did not impair the ability of c-Cbl to increase EGFR ubiquitination and degradation in living cells (Figures 6C and 6D). However, a mutant at site 371 completely lost the ability to enhance receptor ubiquitination and degradation in living cells (Figure 6D). Tyrosine 371 flanks the RF of all Cbl proteins, but it is not present in 70Z-Cbl, which explains why this oncogenic variant lacks ubiquitin ligase activity in vitro (Figure 3A) and in living cells (Figure 1). In line with the importance of Cbl phosphorylation at this site, a bacterial form of c-Cbl, whose respective tyrosine was mutated, was inactive in an in vitro ubiquitination assay (Figure 6E). Control experiments confirmed retention of EGFR binding by the mutant form of c-Cbl. In conclusion, two tyrosine phosphorylation events safeguard productive ubiquitination and subsequent degradation of a ligand-activated EGFR. First, the receptor undergoes tyrosine phosphorylation at a consensus Cbl docking site, and then the ubiquitin ligase activity of the recruited adaptor is activated by phosphorylation at a site flanking the RF.

Discussion

Two features are common to all Cbl-associated signaling pathways. First, the function of Cbl is primarily suppressive, and second, all pathways involve tyrosine kinases that physically recruit Cbl and elevate its tyrosine phosphorylation. By developing an in vitro ubiquitination assay of EGFR, the present study suggests that Cbl negatively regulates signaling because it recruits active tyrosine kinases to the ubiquitin-proteasome degradative machinery (Figure 2). In addition, our results provide

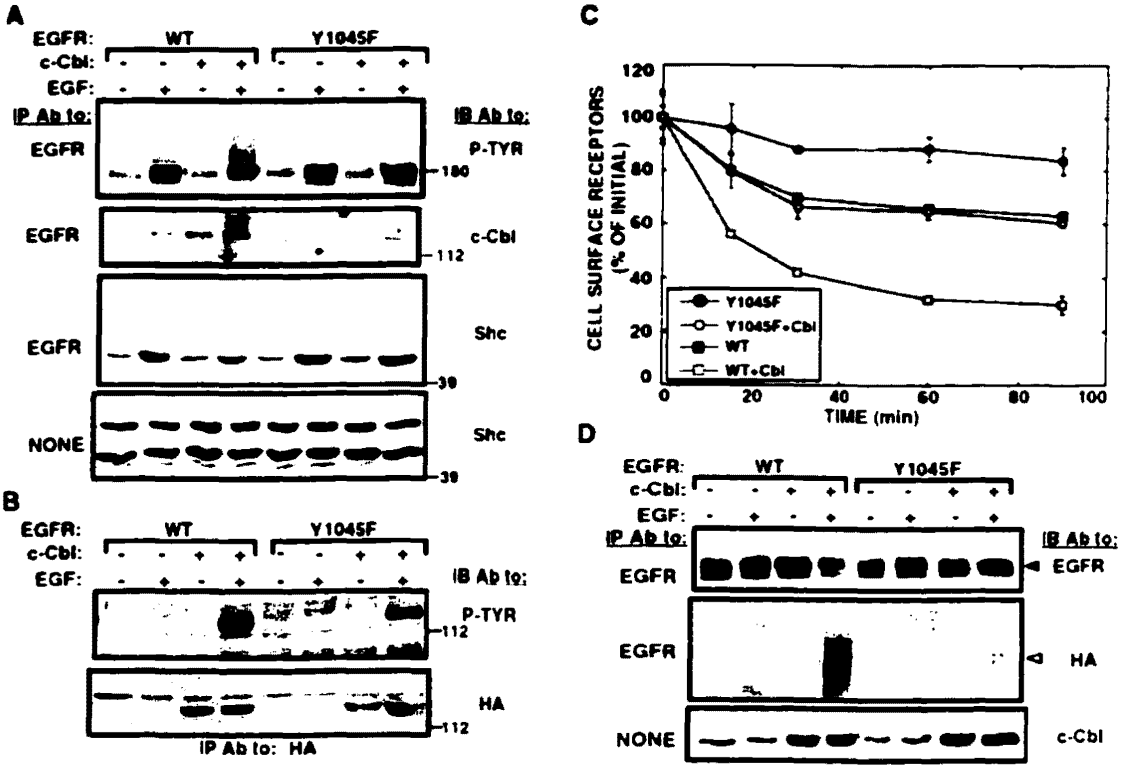

Figure 5. An EGFR Mutated at Tyrosine 1045 Is Impaired in Receptor Downregulation, Ubiquitination, and Degradation

(A) Monolayers of CHO cells were transiently transfected with vectors encoding an HA-tagged c-Cbl or a control empty vector. Cotransfection with plasmids encoding EGFR, either a wild-type receptor or a tyrosine 1045 mutant (Y1045F), was performed as indicated. Following 48 hr of incubation, monolayers were incubated for 10 min at 37°C with or without EGF (100 ng/ml). Whole-cell lysates were subjected to immunoprecipitation with a mAb to EGFR. Subsequent to gel electrophoresis and electrotransfer, filters were immunoblotted (IB) with the indicated antibodies to either phosphotyrosine (P-TYR), Shc, or Cbl. Alternatively, aliquots of the corresponding whole-cell lysates were directly resolved by electrophoresis and then analyzed by using an antibody to Shc.

(B) Cell monolayers were treated as in (A) and the HA-tagged c-Cbl molecules immunoprecipitated by using anti-HA antibodies. Immunocomplexes were resolved by gel electrophoresis and immunoblotting (IB) with the indicated antibodies to phosphotyrosine (P-TYR) or to HA.

(C) Monolayers of CHO cells were transfected with vectors encoding the wild-type form of EGFR or the Y1045F mutant. Along with EGFR plasmids, monolayers were transfected with a c-Cbl-expression vector or a control empty plasmid. Duplicate monolayers were assayed for downregulation of EGFR 48 hr posttransfection.

(D) CHO cells were transiently transfected as in (A) except that a plasmid driving the expression of HA-tagged ubiquitin was included. Following 48 hr, cell monolayers were treated for 10 min at 37°C without or with EGF (100 ng/ml). Receptor degradation and ubiquitination assays were performed as described in the legend to Figure 1B. The lower panel presents immunoblotting of whole-cell extracts with an antibody to c-Cbl. An open arrowhead indicates the location of the ubiquitinated form of EGFR, and a closed arrowhead marks the major unmodified form.

an explanation to the second common feature of Cbl signaling: tyrosine phosphorylation of Cbl is absolutely necessary for its ubiquitin ligase activity toward a substrate tyrosine kinase (Figure 6). Conceivably, the interactions between EGFR and c-Cbl may be summarized in a sequential model (Figure 7). Steps 1 and 2 involve two distinct tyrosine phosphorylation events. Initially, an autophosphorylation reaction creates docking sites for several signaling proteins, including a Cbl binding site at tyrosine 1045 of EGFR. Second, EGFR *trans*-phosphorylates Cbl at a linker domain, which activates an associated ubiquitin ligase activity. Interestingly, only the N-terminal half of c-Cbl (see Figure 3B) is necessary for recruitment of an active E2 and for the ensuing substrate ubiquitination (step 3). This conclusion is consistent with the shorter structures of the two known invertebrate forms of Cbl, as well as with the structure of Cbl-3 (Keane et al., 1999). Below, we discuss two major unresolved aspects of the proposed model. First, we refer to analogous phosphorylation-dependent ubiquitin ligation systems and deal with the mechanism enabling c-Cbl to mediate substrate ubiquitination. Second, we address the possibility that the various steps of EGFR–Cbl interactions take place along the journey of EGFR to the lysosome.

Ubiquitin Ligase Activity of Cbl Proteins

To achieve target specificity, the ubiquitin-proteasome system selects its substrates in a highly regulated manner (Hershko and Ciechanover, 1998). Of the three-step ubiquitin transfer cascade, the E3-mediated ligation step is the most variable and specific. To safeguard selectivity, multiprotein E3 complexes, whose association often involves phosphorylation events, are assembled at the substrate. The potential role of Cbl proteins in such E3 complexes that target activated tyrosine kinases to degradation may be illuminated by the analogy to one of the best characterized modular E3 complexes

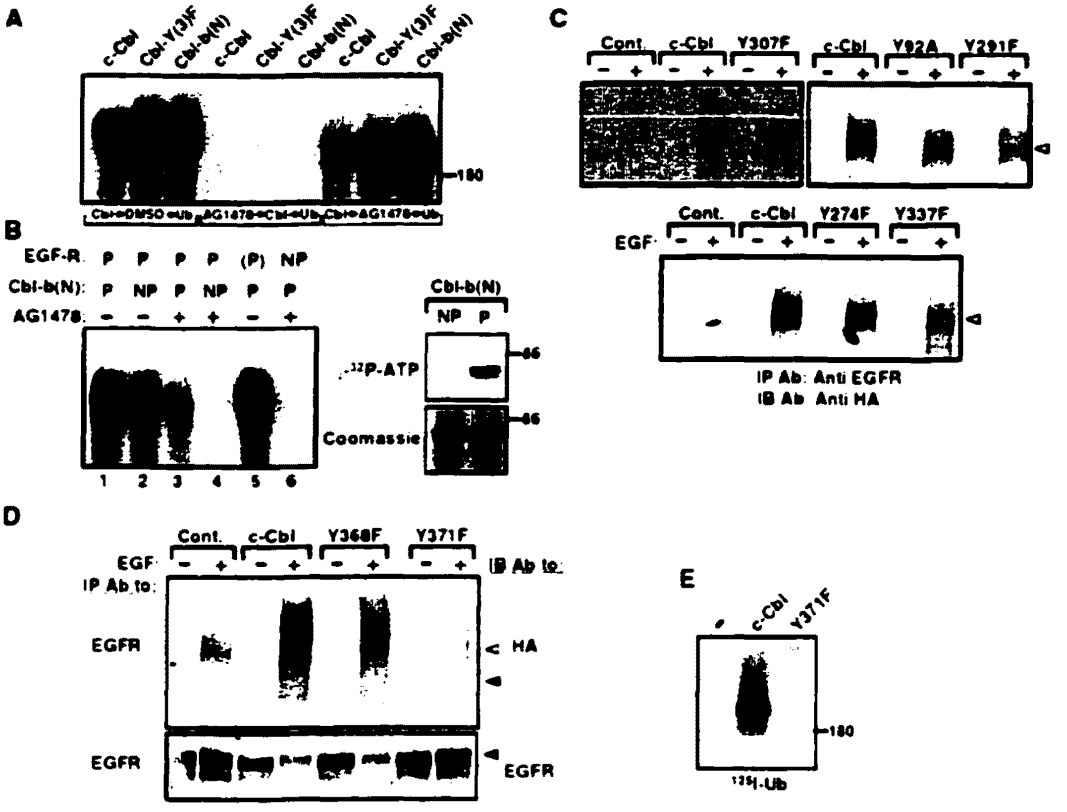

Figure 6. Tyrosine Phosphorylation of Cbl Proteins Is Essential for EGFR Ubiquitination In Vitro and in Living Cells

(A) An in vitro ubiquitination reaction of EGFR was performed with the indicated GST–Cbl proteins. An immunopurified EGFR was first preincubated for 20 min at 4°C with ATP under conditions that allow receptor autophosphorylation, and then we added either AG1478 (50 μM, or solvent dimethylsulfoxide [DMSO] for control) or a GST–Cbl protein. The order of adding the kinase inhibitor and the Cbl proteins is indicated at the bottom.

(B) GST–Cbl-b(N) was immobilized on gluthatione–agarose and subjected to phosphorylation (marked by P letters) by preincubation with a membrane preparation from A-431 cells. Likewise, EGFR was used either in its phosphorylated (P) or nonphosphorylated (NP) form and mixed with aliquots of Cbl-b(N). AG1478 (50 μM) was added as indicated prior to an in vitro ubiquitination reaction. In the lane labeled by (P), EGFR was not subjected to prephosphorylation, but kinase activity was not inhibited (lane 5). To verify in vitro phosphorylation, Cbl-b(N) was similarly treated in the presence of radioactive ATP (right panel). Dye staining of the corresponding lanes is also shown.

(C) CHO cells were cotransfected with an EGFR expression vector and a plasmid encoding a hemagglutinin (HA)–tagged ubiquitin, along with plasmids encoding the indicated Cbl proteins. As control, we used an empty expression vector (Cont.). Each monolayer was split into two separate plates 24 hr after transfection, and 24 hr later identical sister plates were incubated for 15 min at 37°C without or with EGF (100 ng/ml). Cell lysates were subjected to immunoprecipitation (IP) with an anti-EGFR antibody, followed by immunoblotting (IB) with an antibody directed to HA.

(D) A ubiquitination assay of EGFR in living cells was performed as described in (C), except for the use of plasmids encoding c-Cbl proteins mutated at tyrosines 368 or 371. The lower panel shows the results of immunoblotting with an anti-EGFR antibody

(E) An in vitro ubiquitination assay of EGFR was performed in the presence of the indicated mutant Cbl proteins.

of the SCF (Skp1-Cdc53/Cul1-F box) type (Maniatis, 1999). Both IκB, an inhibitory subunit of the NF-κB transcription factor, and the transcriptional coactivator β-catenin are recognized by a receptor called β-TRCP (Yaron et al., 1998). This receptor binds the substrates through its WD40 domain, which recognizes vicinal phosphoserine residues. Another domain, the F box, binds a second component of the SCF, Skp1, which recruits a third subunit, Cdc53/CUL1 (reviewed in Maniatis, 1999). Similar modular complexes mediate degradation of a variety of other proteins, including mitotic cyclins and hypoxia-induced proteins (reviewed in Tyers and Willems, 1999), all sharing a recently identified fourth subunit, Rbx1/Apc11/Hrt1, which encompasses an RF (Seol et al., 1999, and references therein). Like β-TRCP and other receptor subunits of E3 complexes, Cbl proteins physically associate with their ubiquitination substrates in a phosphorylation-dependent manner. Therefore, we raise the possibility that Cbl is functionally equivalent to a combination of β-TRCP and Rbx1. Our finding that the segment linking the SH2 and RF domains of Cbl must be modified suggests a conformational change that activates the E3 complex, in analogy to the Rbx1 component of the VHL-type E3 complex (Kamura et al., 1999).

While it is presently unknown whether or not Cbl recruits E3 ancillary proteins in a phosphorylation-dependent manner, it seems likely that the RF mediates recruitment of the E2 enzyme. This assumption is based upon the occurrence of RF domains in many complexes that mediate substrate ubiquitination (Tyers and Willems, 1999), and the recently observed direct binding of an

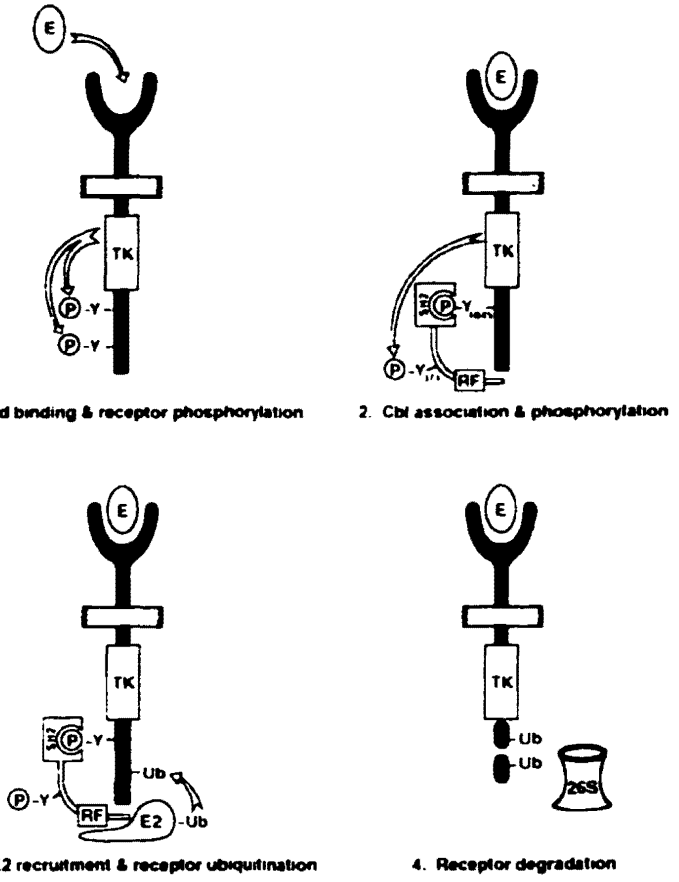

Figure 7. Proposed Sequential Process Leading to EGFR Degradation

Binding of EGF (E) to the extracellular part of EGFR stimulates the tyrosine kinase (TK) domain and results in elevated phosphorylation of several C-terminally located tyrosines, including tyrosine-1045 (step 1). The latter serves as a docking site for the SH2 domain of c-Cbl. Once a stable EGFR Cbl complex is formed (step 2), the adaptor protein undergoes phosphorylation at a region linking the SH2 and the RING finger (RF) domains. This phosphorylation event allows, through an unknown mechanism, recruitment of a ubiquitin-loaded E2 molecule (step 3). Then, E2 relays its thiolester-bound ubiquitin to EGFR, thereby enabling recruitment of the 26S proteasome, and proteolysis of the cytoplasmic portion of EGFR (step 4). Apparently, the four steps occur while the receptor translocates from the plasma membrane, via an endosomal compartment, to a prelysosomal structure, where the luminal ectodomain is exposed to lysosomal hydrolases.

E2, called UBCM4, to a set of RF proteins (Martinez-Noel et al., 1999). Despite the unknown functions of Cbl phosphorylation, it is clear that the Cbl-associated ubiquitin ligase activity is essential for desensitization of signaling processes. One exemplification is provided by oncogenic strategies that apparently corrupt the normal process of EGFR degradation: the essential phosphorylation site of Cbl at tyrosine 371 is deleted in the oncogenic 70Z-Cbl form, and the viral *cbl* gene encodes only an SH2 domain without an RF. On the other hand, the site of EGFR that mediates Cbl binding is frequently deleted in erbB oncogenes of avian erythroblatosis virus strains (Lee et al., 1993). Deletion of this site may inhibit inactivation of the virally encoded form of EGFR and thereby prolong cellular activation. Consistent with the transforming effect of blocking EGFR–Cbl interaction, the respective artificial mutants of either Cbl (Andoniou et al., 1994) or EGFR (Lee et al., 1993; Traverse et al., 1994) are characterized by enhanced signaling.

Relationships between Endocytosis and Cbl-Induced Ubiquitination of Activated Receptors

Ubiquitin conjugation to substrate proteins almost invariably targets them to degradation by the proteasome (Hershko and Ciechanover, 1998). Accordingly, proteasome inhibitors block intracellular degradation of several growth factor receptors, whose ligands induce elevated ubiquitination. Included in this list are EGFR (Levkowitz et al., 1998) and the receptor for PDGF (Mori et al., 1995). An alternative role for receptor ubiquitination originally emerged from studies of the yeast α factor receptor (Hicke and Riezman, 1996). Ubiquitination of this receptor plays a causative role in its endocytosis. The situation in mammalian cells is less clear: preventing internalization of the growth hormone receptor abolished its ubiquitination (Govers et al., 1997), and Cbl ' ' macrophages, which are defective in ubiquitination of the colony stimulating factor-1 receptor, retain endocytosis of the ligand (Lee et al., 1999). Identification of tyrosine 1045 of EGFR as the c-Cbl docking site (Figure 4) enabled us to approach the relationships between ubiquitination and receptor endocytosis.

An EGFR mutant whose interaction with c-Cbl was defected exhibited no ubiquitination and degradation, and it apparently remained at the cell surface following stimulation with EGF (Figure 5). However, closer analyses of receptor's fate and ligand degradation revealed that the mutated receptor underwent internalization, that was followed by release of the ligand and recycling back to the cell surface (H. W. et al., unpublished data). Preliminary morphological analyses suggest that recycling occurs already from the early endosome. Because degradation of EGFR is mediated primarily by lysosomal hydrolases (reviewed in Sorkin and Waters, 1993), it seems likely that ubiquitination of EGFR plays a critical role in sorting of internalized receptor molecules to the lysosome. By using other EGFR mutants (Levkowitz et al., 1998) and an RF-defective form of c-Cbl (Waterman

et al., 1999), we have previously localized the sorting effect of c-Cbl to the transition from early to late endosomes. This conclusion is in agreement with several other observations. First, progressive deletions from the carboxyl terminus of EGFR identified residues 1022–1123, a segment that includes tyrosine 1045, as a lysosomal targeting motif (Kornilova et al., 1996). When deleted, the truncation mutant exhibited enhanced recycling and a minimal extent of sorting to the late endosome. Second, a kinase-defective mutant of EGFR, which is devoid of ubiquitination (Figure 4B), slowly internalizes but recycles back to the cell surface upon reaching the early endosome (Felder et al., 1990). It is interesting to note that the Cbl-docking site we identified is found in two ErbB proteins (ErbB-1 and ErbB-2) that undergo ubiquitination and lysosomal degradation (Galcheva Gargova et al., 1995; Mimnaugh et al., 1996). However, it is absent in ErbB-3, a receptor that undergoes no ubiquitination and is constitutively recycled from early endosomes to the plasma membrane (Waterman et al., 1998). Taken together, these lines of evidence support the possibility that ubiquitination of EGFR targets incoming receptors to the late endosome, a compartment where both proteasomal and lysosomal hydrolases may respectively degrade the cytoplasmic and exoplasmic domains.

In summary, the present report identifies a novel activity of Cbl proteins that enables them to assemble the ubiquitination machinery at an activated tyrosine kinase, while sparing the nonstimulated form of growth factor receptors. Previous works have demonstrated that serine phosphorylation of the substrate (e.g., IκB and β-catenin [Aberle et al., 1997]) or the E3 complex itself (e.g., the cyclosome [Lahav-Baratz et al., 1995]) can strictly regulate ubiquitin ligation and subsequent degradation. Cbl-mediated degradation of EGFR presents a unique example in which tyrosine phosphorylation of both the substrate (EGFR) and the E3 complex (Cbl) regulates ubiquitin ligation. Future studies will address the existence of putative additional components of the Cbl-containing E3 complex and the role it plays in targeting endocytosed EGFRs to specific vesicular compartments.

Experimental Procedures

Materials and Antibodies

Radioactive materials were purchased from Amersham (Buckinghamshire, United Kingdom). Iodogen was from Pierce. MG123 was from Calbiochem. AG1478 was a gift from A. Levitzki (Hebrew University, Jerusalem, Israel). An antibody to the Shc protein was purchased from Transduction Laboratories. Anti-hemagglutinin (HA) monoclonal antibody 12CA5 and yeast hexokinase were purchased from Boehringer-Mannheim (Mannheim, Germany). Murine monoclonal antibody (mAb) SG565 to EGF receptor was generated in mice that were immunized with a recombinant extracellular portion of human EGFR. For immunoblot analysis of EGFR, we used a polyclonal antiserum from Santa Cruz Biotechnology (Santa Cruz, CA). Synthetic phosphotyrosine peptides with the following sequences were prepared by using standard procedures: KEDSFLQRpYSSDP TGALTED (EGFR) and IDIFSDpYANFKAKKK (protein tyrosine phosphatase epsilon, a gift from A. Elson).

cDNA Constructs and Expression of Recombinant Fusion Proteins

Mammalian expression plasmids for EGFR, c-Cbl, 70Z-Cbl, Cbl-b, and Cbl-3 (long and short forms) were previously described (Levkowitz et al., 1998; Ettenberg et al., 1999; Keane et al., 1999). The Y1045F mutant of EGFR was prepared by mutagenizing a single strand template. Bacterial GST–Cbl and a GST-70Z-Cbl expression vector were generated by replacement of Cbl's first ATG codon with a BstpEII site and insertion of a BstpEII–SalI fragment into the compatible XmaI and XhoI sites of pGEX-4T2 (Pharmacia). Other GST–Cbl proteins were made in bacteria by constructing similar pGEX vectors. The RF mutant of Cbl (C381A) was described (Waterman et al., 1999). GST–Cbl fusion proteins were affinity purified as we previously described (Levkowitz et al., 1996). Cloning and expression of UBC-H5B and UBC-H5C were described elsewhere (Jensen et al., 1995). Recombinant E1 was produced in Baculovirus-infected Sf-9 cells, purified on an ubiquitin–agarose column, and eluted with adenosine monophosphate.

Receptor Downregulation and Ubiquitination Assays

Receptor downregulation assays were performed as described (Levkowitz et al., 1998). The ubiquitinated form of EGFR was detected in immunoprecipitates prepared from cells that were cotransfected with a plasmid encoding an HA–tagged ubiquitin (a gift from Dirk Bohmann, EMBL, Heidelberg, Germany) and an EGFR expression vector. The receptor was immunoprecipitated from whole-cell lysates, and its ubiquitination levels were determined by immunoblotting with anti-HA antibodies.

In Vitro Assays of Cbl Binding, Receptor Ubiquitination, and Phosphorylation

EGFR was immunoprecipitated from cleared lysates of A-431 cells by using an agarose-immobilized mAb SG565 as described (Waterman et al., 1999). Following purification, agarose beads were extensively washed and resuspended in buffer containing 40 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 2 mM DTT, 2 mM ATP-γ-S, and 3 μg/ml ^{125}I-labeled ubiquitin (or 0.4 mg/ml unlabeled ubiquitin). To deplete endogenous ATP, hexokinase (1 mg/ml) and 2-deoxyglucose (20 mM) were also included. Crude rabbit reticulocyte lysate (5 μl, from Promega) or the previously described (Abu Hatoum et al., 1998) fraction I (250 μg), fraction IIA (50 μg), purified E1 (2 μg), or recombinant E1 (1 μg) and E2 (7 μl of crude bacterial extract) were added as indicated. Reaction mixtures were supplemented with a GST–Cbl protein (5 μg) and incubated for 1 hr at 30°C. The beads were then extensively washed and EGFR eluted with gel sample buffer. In vitro phosphorylation of an immunoprecipitated EGFR was performed by using a 20 min long incubation on ice with buffer containing 20 mM HEPES-HCl (pH 7.5), 150 mM NaCl, 0.1% Triton X-100, 10% glycerol, 10 mM $MnCl_2$, and 5 μCi γ-^{32}P-ATP. Immunoprecipitates were then washed and resolved by gel electrophoresis. To test binding of Cbl proteins to EGFR, we prepared whole lysates from EGF-stimulated A-431 cells and incubated them for 1 hr at 4°C with immobilized GST–Cbl proteins. Thereafter, the associated EGFR was detected by gel electrophoresis and immunoblotting.

In Vitro Degradation Assay of EGFR

Cell-free degradation was performed following EGFR conjugation with a radiolabeled ubiquitin in a reaction mixture containing a purified 26S proteasome preparation (1 μg) (Ben-Shahar et al., 1999) in buffer containing 40 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 2 mM DTT, 4 mM ATP, 2 mg/ml ovalbumin, 20 μg/ml ubiquitin-aldehyde and a broad-specificity protease inhibitor cocktail (Calbiochem). ATP depletion and inhibition of proteasomal activity were respectively attained by preincubation of the proteasome preparation for 10 min at 37°C with either hexokinase (1 mg/ml) and 2-deoxyglucose (20 mM) or with MG132 (12.5 μM).

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